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STUDIES ON PENETRATION OF DYES WITH GLASS ELECTRODE

IV. Penetration of Brilliant Cresyl Blue into Nitella flexilis

By MARIAN IRWIN

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Accepted for publication, June 19, 1930)

I

INTRODUCTION

Although basic dyes play a special rôle in biology, because many of them are readily taken up by living cells, the form in which they penetrate has never been satisfactorily determined.

A basic dye is usually obtained in the form of the salt some of which may be transformed in aqueous solution to free base (the amount depending on the "apparent" dissociation constant of the dye and the pH value), and in the range of pH values generally employed with living cells (approximately between pH 5 and 9.5) both forms of dye must be considered. In this range methylene blue, for example, exists only as a salt. It is very strongly basic[1] so that even at pH 9.5 no free base is formed. Lauth's violet, on the other hand, is less basic, with a basic dissociation constant[1] of 1.9×10^{-3}.

Since the nature of the transformation of the dye salt into the free base is still in dispute[2] the structure of the salt and free base of the dye will not be discussed.

Furthermore, the dissociation constant of cresyl blue has not been determined by such methods as the oxidation-reduction potential or the glass electrode, so that the basicity of the dye is not accurately known.

It is, however, possible to determine approximately the relative ratio of the dye in form of free base/salt by shaking the chloroform with the dye solution and de-

[1] Clark, W. M., Cohen, B. C., and Gibbs, H. D., *Hygienic Laboratory Bulletin*, 1928, No. 151, 202–206.

[2] Henrich, F., Theories of organic chemistry, translated by John Johnson and Dorothy Hahn, London, 1922.

termining colorimetrically the "apparent" partition coefficient $k_{ap} = \frac{C_1}{C_2}$, where C_1 is the concentration of free base of the dye in chloroform and C_2 the concentration of the total dye (free base in equilibrium with the salt) in the aqueous solution. Such a determination was previously made[3] and it was found that the values of the k_{ap} increased with rise in the pH values as if the basic dissociation constant of the dye were about $10^{-5.6}$. But owing to the extreme solubility of the free base in chloroform and to the progressive alteration in the color of the dye in the aqueous solution from about pH 10 upward, it was not possible to determine the value of the true partition coefficient $k_{tr} = \frac{C_3}{C_4}$ where C_3 represents the concentration of free base of the dye in chloroform and C_4 the same in the aqueous solution. The pH value at which the dye existed almost entirely in form of free base was assumed and the value of k_{tr} was accordingly calculated. The dissociation constant was then calculated on basis of this assumption so that it may prove to be different when the determination is made with a more reliable method. But the significance of this observation does not lie in the accurate determination of the dissociation constant but in the proof that the relative increase in the values of k_{ap}, showing roughly the relative ratio of free base/salt, more or less corresponds to the relative increase in the rate of penetration of the dye into *Nitella* as the pH value of the aqueous dye solution is raised.

It has been suggested that basic dyes penetrate living cells in the form of free base[4] and not in the form of salt,[5] but this, despite many attempts, has never been really proven. The rate of penetration of a basic dye has been found to increase with a rise in the pH value of the external dye solution. This observation might be interpreted as showing penetration in the form of free base since the concentration of this form of dye increases as the external pH value rises. But it might be held to indicate that the dye penetrates as positive ions since with a rise in the external pH value an increase of the negative charges in the cell surface might occur which might result in greater penetration of the positive dye ions.

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[3] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, **23**, 251; *J. Gen. Physiol.*, 1925–26, **9**, 561.

[4] The following authors assume that basic dyes penetrate as free base. Overton, E., *Jahrb. wissensch. Bot.*, 1900, **43**, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, **10**, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, **4**, 1. McCutcheon, M., and Lucké, B., *J. Gen. Physiol.*, 1923–24, **6**, 501. Irwin, M., *J. Gen. Physiol.*, 1925–26, **9**, 561.

[5] The following authors account for the penetration of basic dyes through combining of dye cations with protoplasmic constituents. Bethe, A., *Biochem. Z.*, 1922, **137**, 18. Rohde, K., *Arch. ges. Physiol.*, 1920, **182**, 114. Pohle, E., *Deutsch. med. Wochn.*, 1921, **47**, 1464. Collander, R., *Jahrb. wissensch. Bot.*, 1921, **60**, 354.

If the dye penetrates as free base and combines[6] with the sap to form salt which cannot readily escape from the vacuole, an increase in the pH value of the sap might be expected to decrease the rate of penetration. When the pH value of the sap of *Nitella* was increased by entrance of ammonia, the rate of penetration of cresyl blue was decreased[7] from which it was concluded[7] that the dye penetrated as free base. If such a result were indicative of the penetration of the dye as free base we should expect that in decreasing[8] the pH value of the sap an increase in the rate of penetration of cresyl blue would take place. But on lowering the pH value of the sap, through the entrance of acetic acid, a decrease[8] in the rate of penetration was observed, instead of an increase. This decrease may be due to an inhibitory effect of the acid on the protoplasm irrespective of the changes in the sap since various[9] other substances produce an inhibiting effect without penetrating the vacuole.

It is therefore desirable to find a satisfactory way of distinguishing between the penetration of the free base and that of the dye salt. This can be done by observing the effect of the dye on the pH value of the sap. The hydrogen electrode cannot be utilized, owing to the poisoning of the electrode by the dyes and to their oxidation-reduction potential, and the glass electrode is therefore employed. Since this requires the use of about 0.4 cc. of sap it is necessary to choose large cells which yield sap in sufficient amount for analysis. This can easily be collected from the vacuoles of cells of *Nitella*. Its behavior toward dyes is roughly comparable to that of such cells as *Paramecium*, *Spirogyra*, *Elodea*, and *Valonia* so that by studying the mechanism of dye penetration with *Nitella* we may gain some insight into the behavior of other cells as well.

In the present paper therefore determinations of the pH values of the sap of *Nitella* by the glass electrode[10, 11] are used as a means of studying the form of dye penetrating the vacuoles of living cells.

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[6] E. N. Harvey was the first to suggest that the free base was converted to the salt in the sap. See reference under footnote 4.

[7] McCutcheon, M., and Lucké, B., *J. Gen. Physiol.*, 1923–24, **6,** 501.

[8] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, **24,** 54; *J. Gen. Physiol.*, 1926–27, **10,** 271; 1927–28, **11,** 111.

[9] See footnote 8, and also Irwin, M., *J. Gen. Physiol.*, 1926–27, **10,** 425.

[10] Through the kindness of Dr. D. A. MacInnes and of Dr. M. Dole a glass electrode apparatus was devised with which it was possible to measure a very small amount of liquid. Through their cooperation it was possible to determine whether this method was applicable to the studies of the penetration of cresyl blue into *Nitella* cells, as mentioned in their article (MacInnes, D. A., and Dole, M., *J. Gen. Physiol.*, 1928–29, **12,** 805).

[11] Applicability of the glass electrode methods to the measurement of the sap, mentioned under footnote 9, was described separately by Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27,** 132. Preliminary report of the present paper was published, *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27,** 991.

II

Methods

Solutions Employed.—All buffer solutions were made up according to Clark's[12] standards and diluted ten times. The pH value of these solutions was checked by means of the glass electrode. The dyes were in all cases dissolved in these dilute solutions.

Condition of the Dye Used.—The cresyl blue was purchased as a salt. The brilliant cresyl blue (called cresyl blue in the text) was manufactured by Grübler previous to 1914, and appears to contain (in the range of pH values between pH 5.5 and pH 9.2) chiefly cresyl blue in form of salt or free base. It also contains another dye, *X*, but in the solutions employed the amount is so small in comparison to the cresyl blue (free base or salt) that *X* may be neglected. The difference in behavior between the cresyl blue and *X* is well marked so that it can be readily distinguished. Cresyl blue is absorbed as free base by the chloroform and appears orange. This orange dye can be entirely removed from the chloroform on shaking the chloroform with the artificial sap of *Nitella* (0.1 N KCl at pH 5.5) in which it appears blue. On shaking the cresyl blue solution with chloroform, the dye is absorbed much more from pH 9.2 than from pH 5.5, showing that the free base of cresyl blue is much more soluble in chloroform than the dye salt, and that the ratio of free base/salt of cresyl blue increases with the rise in the pH value. The dye *X*, on the other hand, appears pinkish red in chloroform. The amount of *X* absorbed by the chloroform is so much less than that of the free base of cresyl blue that it becomes visible only when the experimental condition is favorable (*i.e.*, at a high concentration and low pH value). For example, on shaking 0.07 per cent cresyl blue solution at pH 5.5 with the chloroform, the dye appears red in chloroform. On shaking with artificial sap, the red dye is not removed from the chloroform; in fact only a trace of the blue dye appears in the sap, which may very well be a trace of the cresyl blue extracted from the chloroform. On shaking 0.005 per cent cresyl blue solution at pH 9.2 or at pH 5.5, or 0.07 per cent cresyl blue solution at pH 9.2, or 0.025 per cent cresyl blue solution at pH 6.8 with chloroform, the dye absorbed appears orange in chloroform. But when this chloroform is shaken with the sap, until the orange dye is completely removed from the chloroform, the red dye becomes visible in the chloroform, and is not extractable by the sap from the chloroform. This red dye is absorbed by the chloroform to about the same extent from pH 9.2 or from pH 5.5 cresyl blue solution, but the amount absorbed increases with the rise in the concentration of aqueous cresyl blue solution. The amount of *X* absorbed is so small, however, that the absorption causes only a very slight decrease in the concentration of the total dye solution

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[12] Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 1928.

even when the chloroform is shaken with 0.07 per cent cresyl blue solution at pH 5.5. These experiments are being continued and their results will be reported in later publications.

Spectrophotometric measurements of these solutions of cresyl blue show that they give absorption curves characteristic of cresyl blue.

The free base of cresyl blue was obtained as follows: 0.005 per cent cresyl blue solution at pH 9.2 was shaken with chloroform. The chloroform was then carefully freed from the aqueous solution and immediately placed in a pyrex glass vial and caused to evaporate rapidly by bubbling air through the solution. The freshly collected sap was then placed in this glass vial, and the concentration of the dye was determined colorimetrically by comparison with the standard dye solutions of known concentrations. There was a small amount of the dye residue which was not readily soluble in the sap, and which was therefore discarded. The pH value of the sap containing the dissolved dye was at once determined by the glass electrode.

To ascertain whether this dye was contaminated by other alkaline substances absorbed by the chloroform from the buffer solution which might alter the pH value of the sap the following experiments were made. The chloroform was shaken with the buffer solution at pH 9.2 containing no dye and was evaporated in the vial as before. The freshly extracted sap was placed in the vial and the pH value was immediately determined by the glass electrode. Since the pH value of the sap collected in this vial was the same as that of the sap collected in a control vial (which had not come in contact with the chloroform) it is apparent that the chloroform does not absorb any alkaline substance from the buffer solution in the absence of dye which would alter the pH value of the sap. Whatever alteration occurs in the pH value of the sap in presence of the dye must therefore be due to the dye alone.

It may be asked whether this dye is actually free base of cresyl blue. It cannot be a pseudo base, since it is very soluble in water and is readily reversible, forming a salt at lower pH values (appearing blue), while the pseudo base is not readily soluble in water, in which it appears colorless, and is irreversible. On shaking this dye solution (made up in buffer mixtures) with chloroform, the behavior is found to be very much like that of the original cresyl blue solution. The spectrophotometric determination shows that the absorption curve of the dye taken up by chloroform is identical with that of brilliant cresyl blue. It would therefore appear that the dye represents primarily the free base of brilliant cresyl blue. A detailed account of these results will be subsequently published.

Condition of Cells before Experiments.—As previously described,[13] single cells were cut from the central portion of the plant and kept about 20 hours in pans of

[13] Regarding the care of cells and detection of injury see Irwin, M., *J. Gen. Physiol.*, 1925–28, **8**, 147; 1926–27, **10**, 271, 927; 1927–28, **11**, 123; 1928–29, **12**, 147.

tap water at about 20°C. in a well ventilated room in diffused light. The cells which have suffered severe injury at the time of cutting die over night and those which may be slightly injured may recover so as to be suitable for use. The effect of cutting and of the subsequent treatment of the cells as described in connection with electrical measurements[14] is not manifested in the same way in the determination of penetration of dyes into the vacuole, so that it is not necessary, for example, to keep each cell in a separate test-tube for a day or more before an experiment. The precaution, however, was always taken of placing only a few cells in a large pan of water so as to minimize any possible effect of the sap diffusing from the dead cells to the living cells.

Experiments on Penetration.—Cells were placed in solutions with one end (only a very small portion, just enough to avoid contamination) exposed to the air in a moist chamber, at the temperature at which the glass electrode measurement was subsequently made. The cells were kept in the dye solution about 15 minutes. Leaving one end of the cell out of the solution for this period in a moist chamber was not injurious since cells kept in this way in tap water containing no dye even after 8 hours showed no signs of injury.

Extraction of the Sap.—The cells were removed from the solution, rinsed rapidly in distilled water and wiped gently first with a soft dampened cheese cloth and then with the dry cloth. Then each cell was cut open by a pair of scissors (the cut being made at the end which projected out of the solution) and the sap was allowed to flow out into a pyrex glass vial (small enough to prevent evaporation). By thus extracting the sap from the unstained portion of the cell, it was possible to avoid contamination of the sap by dye diffusing out of the cell wall at the time of extraction. Two successive extractions of sap were made from the vacuole of each living cell. The first extraction, called for convenience "sap," was made without pressing on the cell wall, while the second extraction called "sap mixture" was made by squeezing the cell wall vigorously so as to press the protoplasm into the vacuolar sap. The pH value of the sap was found to be about 0.6 pH lower than that of the sap mixture. It took about 15 minutes for one person to collect the sap and the corresponding sap mixture sufficient for analysis. For each reading by the glass electrode method it required about 0.4 cc. of sap collected from the vacuoles of 40 or more cells. The extraction was made at the same temperature as the temperature at which the electrode measurement was made.

Standardization of the Glass Electrode and Method of Measurement.—The apparatus used is identical with the one described by MacInnes and Dole[15] but the method of standardization of the glass electrode and the calculation of the pH value of the unknown solution used was different. The standard solution of the M/20 potassium hydrogen phthalate was made up to pH 3.97 and its pH value was determined by means of the hydrogen electrode. This solution was made up every

[14] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929–30, **13**, 467.
[15] For reference see footnote 10.

2 weeks to keep the pH value constant. Due to the very low temperature coefficient of this solution the alteration in the pH value with varying temperature is negligible in the range in which the experiments were carried out (between 20° and 23°C.).

Before each series of experiments the temperature was noted, and the glass electrode was standardized by determining the potential of the standard phthalate solution[16] at pH 3.97. Then the pH value of the standard M/15 phosphate buffer solution at pH 6.647 (the pH value of which was checked by the hydrogen electrode) was determined by means of the glass electrode to ascertain the behavior of the electrode; in no case did the reading vary more than ±0.03 pH. The potential of the unknown solution was then determined, and the pH value of the unknown solution was calculated for the given temperature according to the usual formula[17]

$$pH_m = pH_s + \frac{E_m - E_s}{\frac{RT}{F}}$$

where pH_m denotes the pH value of the unknown solution and pH_s that of the standard, while E_m represents the potential of the unknown solution and E_s that of the standard (the value of $\frac{RT}{F}$ at different temperatures is given by Clark[11]).

The temperature during the day did not usually vary more than ±0.5°C. but in case a variation greater than ±0.5°C. occurred, the electrode was re-standardized. From day to day the temperature varied between 20° and 23°C., but this variation did not bring about errors, since the glass electrode was always standardized and a temperature correction was made accordingly. After each series of experiments the electrode was again standardized with the phthalate and the phosphate buffer to see whether the electrode remained unaltered during the experiments. In many cases where the test solution is much less buffered than the standard phosphate solution, it may be desirable to check the glass electrode before and after a series of experiments with a standard solution which is buffered to about the same extent as the test solution. This was done in the case of the *Nitella* sap, by using M/150 phosphate buffer solution at pH 5.5, or the control sap, whenever there was a possibility of the effect of the sap in presence of the dye on the electrode in a series of experiments. In the case of all the experiments presented in the text the electrode remained unchanged through each series of experiments, in that there was no variation of more than ±0.05 pH value with these standard solutions.

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[16] For review and bibliography on the glass electrode methods see Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 1928.

[17] The equation is taken from the one described by Kerridge, P. T., *Biochem. J.*, 1925, **19**, 611.

Three electrodes were employed for the experiments described in the text. In no case did the pH value of the standard phosphate buffer solutions determined by these electrodes vary more than ±0.03 pH.

Method of Washing the Glass Electrode when Successive Determinations Are to be Made.—When the glass electrode is not in use, it is kept in 0.1 N HCl. When two measurements with different samples are to be made in rapid succession washing with distilled water (without washing with the acid) is adequate if the test solution is such that it does not measurably affect the electrode or the reading, or if the solution is sufficiently buffered, but if the test solution does affect the electrode and if it is not sufficiently buffered, the alteration will result in a wrong pH value. An example of such cases may be cited as follows. The pH value of a strongly buffered solution, such as standard phosphate buffer at pH 6.647, in presence or absence of basic dye, or that of the distilled water containing no dye, may be measured repeatedly without showing any effect of the test solution on the electrode whether the electrode is washed after each measurement with 0.1 N HCl and then with distilled water or only with distilled water. But if the distilled water contains, for example, 0.07 per cent cresyl blue in form of salt or free base, an effect on the electrode is manifested by an increase in the pH value after each successive measurement if the electrode is washed only with distilled water each time. But such an effect is not manifested if the electrode is first washed with acid each time before applying distilled water.

The freshly extracted sap is more buffered than the distilled water but not sufficiently so to enable us to omit the washing with acid each time, even though the electrode is always washed with the test sap before each measurement. If the electrode is not washed with the acid there is a small but progressive increase in the observed pH value of sap containing cresyl blue (in form of salt or in form of free base) with each successive drop of the same sample of sap taken for each one of the five measurements, where the maximum increase is a little over 0.1 pH. The observed pH value of the same sap remains constant throughout such a series of experiments when the electrode is washed with 0.1 N HCl and then with distilled water followed by final washing with test solution after each measurement. There is no danger of the acid diffusing out of the electrode which has been washed with acid into the sap, since the pH value of the sap containing no dye is found to be the same whether the electrode is first washed with the acid or not.

To avoid the errors arising from this source, the following procedure was adopted in the experiments described in the text. Before using the electrode it is removed from 0.1 N HCl, wiped with filter paper, washed in distilled water, and wiped again. Then the electrode is washed quickly in the solution, the potential of which is to be determined. After measurement, the electrode is washed in distilled water, wiped, and placed in 0.1 N HCl until the effect of the test solution on the electrode is completely removed. When the electrode is not in use, it is always kept in the 0.1 N HCl. The temperature of the solutions used to wash the electrode is kept within ±0.5°C. of the temperature of the test solution.

Time Curve for Glass Electrode Measurement.—It is always necessary to continue measurement for about 5 or 10 minutes to see if there is any change in the potential with time. Such a change may occur from the effect of the solution on the electrode or from the change in the solution proper. The results show that there is no alteration in the pH value of the buffer solution at pH 6.65 within 5 minutes, nor in the pH value of the control sap. There is a tendency for the pH value of the sap containing the dye to rise with time, but the maximum rise in 5 minutes is only 0.05 pH, which is within an experimentl error. The two measurements made within 2 minutes agree within ±0.03 pH; the measurements given in the text represent the average of two readings made, (*a*) one-half minute after the electrode has first come in contact with the test solution, and (*b*) one minute and a half later.

The pH Value of the Control Sap or the Sap Mixture. If the pH value of the control sap changed rapidly after extraction, it would interfere with such experiments as are described in the text, but fortunately there was no alteration in the pH value of the sap nor of the sap mixture even on standing in a pyrex glass vial (stoppered) 2 hours after extraction. Even on keeping the sap in the refrigerator for 20 hours the pH value of the sap was found to increase only 0.2 pH.

Furthermore there was practically no alteration in the pH value of the sap nor of the sap mixture during the 15 minutes required for extraction. This test was made colorimetrically,[18] since by this method only a small capillary tube of sap was sufficient so that it was possible to complete the first determination only 3 minutes after the extraction had begun. The indicator method was found to be adequate since the pH value of the sap determined by this method agreed closely enough (within ±0.1 pH) with the pH value determined by the glass electrode.

From day to day there was some variation in the pH value of the control sap, the maximum variation being about ±0.2 pH. Owing to this an alteration in the pH value of the sap less than 0.2 pH should not be taken seriously. Though the pH value of the sap did not alter on standing for several hours, the measurement with the glass electrode was always made immediately after the sap was extracted.

Each reading given in the text is an average of 10 to 15 experiments employing altogether 400 cells or more.

III

Nitella flexilis (*A*)

The Sap.—The average sap of the control cells (taken from tap water) is at about pH 5.36 (Table I).

When the cresyl blue salt at a concentration in the neighborhood of 0.07 per cent is dissolved in the sap *in vitro* the pH value of the sap

[18] For method see Irwin, M., *J. Gen. Physiol.*, 1925–26, **9**, 240, Section III.

is found to decrease by about 0.07 pH, which is too small an alteration to be considered seriously (Table I).

When about 0.07 per cent free base [19] of cresyl blue is dissolved in the sap *in vitro*, the pH value is found to increase by about 0.55 (Table I).

TABLE I

pH Value of the Sap of Living Cells of Nitella flexilis (A)

In presence of cresyl blue. Average temperature 21°C. Electrode I was used. Unless otherwise stated, penetration experiments were carried out with one end of the cell outside the solution in moist chamber, to avoid contamination of the sap. The duration of the experiments was about 15 minutes.

	Conc. of dye in sap in per cent	pH of sap	Increase in pH of sap over the control sap	Conc. of dye in sap containing protoplasm in per cent	pH of sap containing protoplasm	Increase in pH of sap containing protoplasm over the control sap containing protoplasm
Control cells in tap water	0	5.36		0	5.97	
Dye salt added *in vitro*	0.069	5.29				
Free base of dye added *in vitro*	0.071	5.91	0.55	0.070	6.48	0.51
Penetration from 0.002 per cent dye at pH 9.2	0.073	5.86	0.50	0.065	6.45	0.48
Penetration from 0.025 per cent dye at pH 6.8	0.071	5.87	0.51	0.074	6.37	0.40
Penetration from 0.004 per cent dye at pH 9.2	0.18	6.66	1.30			
Free base added *in vitro*	0.16	6.50	1.24			
Penetration from 0.002 per cent dye at pH 9.2, when cells are completely immersed	0.070	5.88	0.52			
After 8 hours in tap water containing no dye	0	5.40		0	6.00	
After 1 hour in buffer solution at pH 9.2, containing no dye	0	5.37		0	5.94	

These results prove that the two forms of the dye are distinguishable by determining the pH value with the glass electrode, the dye salt having very little effect on the sap, the free base of the dye distinctly raising the pH value of the sap. If therefore the same amount of dye penetrating the vacuole raises the pH value to about the same extent

[19] For method see Section II in the text.

as the free base of the dye, we may conclude that the dye penetrates as free base and not as salt. The following results show this to be the case.

When cells are placed[19] for about 15 minutes in 0.002 per cent dye solution at pH 9.2 until about 0.07 per cent dye has accumulated in the vacuole, the pH value of the sap is found to increase about 0.5 over that of the control sap (Table I). This increase[20] agrees closely with the increase obtained by dissolving 0.07 per cent free base of the dye in the sap (Table I), thus indicating that the dye penetrates chiefly as free base and not as salt.

Previous experiments[21] have shown that at pH 9.2 cresyl blue exists predominantly as free base so that it would not be surprising if the dye penetrated as free base from this solution. But at a lower pH value, where the dye salt predominates, does the dye enter as salt or as free base? To test this point the dye was allowed to penetrate for about 15 minutes from 0.025 per cent cresyl blue solution at pH 6.8 (in which there was a preponderance of the dye salt).[21] As shown in Table I, 0.07 per cent dye penetrating from this solution has brought about approximately the same increase (about 0.51 pH) in the pH value as 0.07 per cent dye penetrating from the solution at pH 9.2. These results prove that irrespective of the ratio of the free base to salt of the dye in the external solution, the dye penetrates as free base and not as salt.

The dye was furthermore allowed to penetrate to a still higher concentration (0.18 per cent) from 0.004 per cent cresyl blue solution at pH 9.2 in about 15 minutes, to see if further increase in the pH value of the sap would occur with a higher concentration of the dye in the sap. An increase[20] of about 1.30 pH over the control was found to take place, which agreed closely with the rise in the pH value by dissolving 0.16 per cent free base of cresyl blue in the sap *in vitro* (Table I).

The pH value of the sap in the presence of a lower concentration of

[20] These results prove that the alteration in the rate of entrance and exit of the dye formerly reported to occur in the cell after penetration of dye are not due to the production of acid in the sap to the extent of lowering the pH value of the sap below the normal. Irwin, M., *J. Gen. Physiol.*, 1925–28, **8**, 147; *Proc. Soc. Exp. Biol. and Med.*, 1926–27, **24**, 247.

[21] For reference see footnote 3.

cresyl blue (below 0.07 per cent) was determined but owing to the variation in the pH value of the control sap (to the extent of 0.2 pH) it is not desirable to draw conclusions from the lower concentration of the dye in the sap which brought about this increase in the pH value of the sap.

Owing to the fact that these experiments were carried out when a portion of the cell was exposed to the air in a moist chamber (to avoid contamination of the sap from the stained cell wall at the time of extraction) the question may be raised whether the increase in the pH value of the sap may not be caused by the penetration of the alkaline buffer as a result of injury to the cells under these experimental conditions. This "partial immersion" method, however, is proved to cause no injury in the duration of these experiments, as shown by the following experiments.

When the experiments were repeated by completely immersing the cells in 0.002 per cent dye at pH 9.2 for a little less than 15 minutes until 0.07 per cent dye had collected in the sap, the pH value of the sap was found to increase about 0.52 pH, which agrees closely with the rise brought about when cells were only partly immersed in the solution as previously stated (Table I). In this dye solution at pH 9.2 so little staining of the cell wall occurs (with this length of exposure) that no question of errors arising from contamination of the cell sap from the stained cell wall enters in the case of the cells completely immersed in this solution. If the partial immersion method causes injury, thereby hastening the penetration of the alkaline borate buffer, the rise in the pH value of the sap may be expected to be greater when this method is used than the rise when the cells are completely immersed. Since the rise in the pH value is about the same in both cases, we may conclude that the alteration in the pH value of the sap is not due to injury caused by the partial immersion method.

Furthermore the pH value of the cells which were partially immersed in this way in tap water for 8 hours or in the buffer solution at pH 9.2 (containing no dye) for 1 hour was the same as that of the control sap, thus showing that the partial immersion method does not injure the cells (for the duration of exposure described in the text) enough to affect the pH value of the sap (Table I).

Sap Containing Protoplasm.—The most striking fact is that after the

sap has been allowed to flow out without pressure, if the cell wall is squeezed so as to press the protoplasm into the sap, the pH value of this mixture is found to be about 0.6 pH higher than the sap proper (Table I). This may be chiefly due to the presence of the protoplasm, the pH value of which may be higher than that of the sap. Another possible explanation is that the fluid[22] which may exist in the space between the protoplasmic layer and the cell wall may also be squeezed into the sap and may help to raise the pH value of the sap.

In presence of 0.07 per cent cresyl blue free base the pH value of this mixture is increased about 0.51 over that of the control mixture (Table I). The cresyl blue penetrating from the dye solution at pH 9.2 or at pH 6.8 also raises the pH value of sap containing protoplasm by about 0.4 to 0.5 pH (Table I), thus showing that the dye penetrates chiefly as free base into this mixture. Undoubtedly not all the protoplasm can be squeezed into the sap and the protoplasmic layer is very thin so that in the mixture the sap preponderates over the protoplasm. For this reason it would be difficult to conclude from these results whether the dye penetrates chiefly as free base into the protoplasm as it does into the vacuole.

IV

Nitella flexilis (*B*)

To determine if the penetration of cresyl blue as free base will occur with another sample of *Nitella* obtained in a different locality, the experiments were repeated with cells collected elsewhere which are distinguished for convenience from *Nitella* (*A*) by the term *Nitella* (*B*).

The Sap.—The average pH value of the sap of *Nitella* (*B*) is pH 5.6 (Table II), which is about 0.2 pH higher than the pH value of the sap of *Nitella* (*A*).

On dissolving the 0.086 per cent free base[19] of cresyl blue in this sap *in vitro* the pH value of the sap is found to increase about 0.37 pH (Table II). This increase is about 0.2 pH less than is the case with *Nitella* (*A*). This may mean that the sap of *Nitella* (*B*) is more buffered than the sap of *Nitella* (*A*) (Tables I and II). On

[22] Irwin, M., *J. Gen. Physiol.*, 1926–27, **10**, 75.

adding the dye salt to the sap there is practically no alteration in the pH value of the sap. When about 0.075 per cent dye penetrates the vacuole from the cresyl blue solution at pH 9.2 or 6.8, there is an increase in the pH value of 0.4 or 0.45 pH over that of the control sap (Table II), which agrees closely with the rise in the pH value when the free base of the dye is dissolved in the sap *in vitro*. In this case

TABLE II

pH Values of the Sap of Living Cells of Nitella flexilis (B)

In presence of cresyl blue. Average temperature at 22°C. Electrode I was used unless otherwise stated. The penetration experiments lasted about 15 minutes (with precautions to avoid contamination of the sap).

	Conc. of dye in sap in per cent	pH of sap	Increase in pH of sap over the control sap	Conc. of dye containing protoplasm in per cent	pH of sap containing protoplasm	Increase in pH of sap containing protoplasm over the control sap containing protoplasm
Control	0	5.60		0	6.11	
Free base of dye added *in vitro*	0.086	5.97	0.37	0.077	6.43	0.32
Dye salt added *in vitro*	0.080	5.55		0.085	6.07	
Penetration from 0.002 per cent dye at pH 9.2	0.076	6.00	0.40	0.065	6.35	0.24
Penetration from 0.025 per cent dye at pH 6.8	0.077	6.05	0.45	0.069	6.36	0.25
Penetration from 0.01 per cent dye in tap water	0.076	5.96	0.38	0.072	6.37	0.26
Cells placed for ½ hour in buffer solution at pH 9.2 after cells had been in 0.002 per cent dye at pH 9.2 for about 15 minutes	0.075	5.95	0.35	0.070	6.31	0.20
Penetration from 0.002 per cent dye at pH 9.2 Electrode II	0.079	5.98	0.38	0.070	6.39	0.28
Penetration from 0.002 per cent dye at pH 9.2 Electrode III	0.074	5.95	0.35	0.068	6.36	0.25

the increase in the pH value is a little less (about 0.1 to 0.2 pH) than that of the *Nitella* (*A*).

Thus the results on *Nitella* (*B*) confirm those on *Nitella* (*A*), showing that in both cases the dye penetrates the vacuole chiefly as free base.

The Sap Containing Protoplasm.—The pH value of the mixture of

the sap and the protoplasm extracted as before behaved very much like the mixture of *Nitella* (*A*) in that the pH value of the mixture is raised when the free base of the dye is dissolved in the mixture ***in vitro*** (Table II). On examining the pH values of the mixture after penetration of the dye they are found to be 0.1 to 0.2 pH lower than the corresponding pure sap containing approximately the same amount of dye (Table II). Such results may signify that some dye salt[23] penetrates into the protoplasm or that the pH value of the protoplasm is decreased when the dye penetrates as a result of production of acid by the protoplasm.

Though the results definitely show the penetration of cresyl blue into the sap to be chiefly as free base there is still one more uncertainty to be removed and this is in relation to the possibility of the rise in the pH value of the sap taking place as a result of penetration of an alkaline substance other than the dye owing to the injury caused by the penetration of the dye. Such a possibility is imminent owing to previous[20] results showing changes in the rate of penetration from the normal as a result of penetration of over 0.006 per cent dye. Since the concentration of cresyl blue in the vacuole in the present case is high (about 0.07 per cent), it is necessary to prove that the increase in the pH value of the sap is not due to the penetration of an alkaline substance other than the dye into those cells the condition of which is altered from the normal by penetration of the dye.

Since in all the experiments the length of exposure and the concentration of the dye which has penetrated into the sap are approximately equal, if the increase in the pH value of the sap were due to the penetration of an alkaline substance other than the dye, there should be a greater increase in the pH value of the sap when cells are exposed to the dye solution at pH 9.2 than at pH 6.8 or in tap water at pH 7.7. But this increase is found to be about constant irrespective of the external pH values and the external alkaline buffer concentrations, provided the concentration of dye in the sap is approximately the same (Table II). These results therefore point to the fact that whatever

[23] Experiments on penetration of dye from methylene blue, described in the subsequent paper, show that such a lowering is not due to the penetration of the dye salt, so that in all probability the same is true with the cresyl blue. Irwin, M., *J. Gen. Physiol.*, 1930–31, **14**, 19.

secondary changes that might occur in the cell they do not admit an alkaline substance other than the dye into the vacuoles of these cells.

Another proof is as follows. If the increase of 0.4 pH in the sap over the control sap is not due to the penetration of alkaline buffer as a result of secondary changes, but is due to the penetration of about 0.08 per cent cresyl blue free base from the dye solution at pH 9.2, then by transferring such cells from the dye solution into the buffer solution at the same pH value containing no dye there should be no progressive increase in the pH value of the sap. Such is found to be the case experimentally, as shown in Table II, when even after ½ hour exposure of these cells to the buffer solution at pH 9.2 there is no further increase in the pH value of the sap. But a further increase is found to occur if more dye penetrates the vacuole.

These experiments were made with one electrode (I) only: afterward check experiments were made with several electrodes, which gave about the same results as Electrode I. The results from two other electrodes are recorded in Table II.

The spectrophotometric measurements show that the dye which has penetrated from the cresyl blue solution into the sap or into the sap mixture is identical with the external dye solution as well as the cresyl blue free base dissolved in the sap *in vitro* in that the absorption curves are characteristic of cresyl blue.

V

CONCLUSION

From these results we may conclude that the cresyl blue penetrates chiefly as free base and upon coming in contact with the sap at the vacuolar surface it is at once partly converted into the dye salt. At equilibrium the concentration of free base in the sap is proportional to that in the external solution. Thus the rate of penetration of the dye in form of free base and the concentration of the total dye (free base and salt) in the sap at equilibrium are dependent on the dissociation constant of the dye. For this reason both the rate of penetration and the concentration of the dye in the sap at equilibrium are increased with a rise in the external pH value (which increases the concentration

of free base of the dye) or with a decrease in the pH value of the sap (which increases the conversion of the free base to the dye salt).

It is possible that some of the dye may be converted to something different from the dye salt after the dye has penetrated the vacuole. Previous experiments[20] have shown that as the concentration of the dye in the sap through penetration reached a certain point the rate of penetration progressively increased while that of the exit decreased from that of the normal. Such an alteration in the rate is not due to the excess production of acid in the sap as shown by the preceding results. It may be caused by some kind of change in the dye as the dye penetrates the sap. Such a dye may be either another tautomer of cresyl blue, or a compound formed between the cresyl blue and the sap, or another dye. To affect the rate, as already stated, the reaction producing this dye must be less reversible than that producing cresyl blue salt or this dye must have a lower rate of exit from the vacuole into the external solution than cresyl blue salt.

These results cannot be due to exchange of dye cations for hydrogen ions, as this would involve a lower rate of penetration since the surfaces are non-aqueous and the concentration of hydrogen ions in the sap is too low. This will be discussed in subsequent papers.

SUMMARY

Glass electrode measurements of the pH value of the sap of *Nitella* show that cresyl blue in form of free base penetrates the vacuoles and raises pH value of the sap to about the same degree as the free base of the dye added to the sap *in vitro*, while the dye salt dissolved in the sap does not alter its pH value. It is proved conclusively that the increase in the pH value of the sap is due only to the presence of the dye and not to some other alkaline substance.

Spectrophotometric measurements show that the dye which penetrates the vacuole is chiefly cresyl blue.

When the protoplasm is squeezed into the sap, the pH value of the sap is higher than that of the pure sap. Such a mixture behaves very much like the sap in respect to the dye.

STUDIES ON PENETRATION OF DYES WITH GLASS ELECTRODE

V. Why Does Azure B Penetrate More Readily Than Methylene Blue or Crystal Violet?

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I

The results[1] from measurements made with the glass electrode and spectrophotometer show that brilliant cresyl blue penetrates as free base into the vacuoles of living cells of *Nitella*. The present paper describes similar experiments with azure B, methylene blue, and crystal violet.

Since the methods for the condition of cells, extraction of the sap, penetration experiments, extraction of free base of dyes, making up of dye solutions, and measurements by means of the glass electrode have been published[1] in detail, they are omitted here. The dyes were obtained as salts. The azure B (in pure form) was made by W. C. Holmes of the Color Laboratory, Washington, D. C., methylene blue (medicinal) by Merck and Co., crystal violet by Grübler (obtained before 1914). These dyes affected the electrode slightly more than cresyl blue (causing the pH value of the sap containing the dye to increase between 0.1 and 0.2 pH value after the fifth measurement (when the electrode was not washed with acid), but these effects were entirely eliminated by washing the electrode with acid as already described.[1] Just as with cresyl blue, the readings did not vary within 5 minutes after the electrode was brought in contact with any one of these dyes.

[1] Irwin, M., *J. Gen. Physiol.*, 1930–31, **14**, 1.

II

Azure B or Trimethyl Thionin

A. Nitella (A)

The Sap.[1]—When about 0.07 per cent azure B salt is dissolved in the sap *in vitro* the pH value remains practically the same as that of the control (Table I), but when the same concentration of the dye in the form of free[1] base is dissolved in the sap *in vitro* the pH value is found to be about 1 pH above that of the control sap (Table I). Owing to this difference it is possible to find out which form enters the vacuoles of living cells of *Nitella* by determining the pH value of the sap after penetration.

When cells are placed in 0.01 per cent azure B solution at pH 9.2 for about 15 minutes until 0.07 per cent dye has penetrated the vacuole, the pH value of the sap is found to increase about 1 pH above that of the control sap (Table I).

Since azure B which has penetrated the vacuole increases the pH value of the sap to about the same extent as the same concentration of the dye in form of free base added to the sap *in vitro*, while the dye salt dissolved in the sap *in vitro* does not alter the pH value of the sap, it would seem that the azure B penetrates chiefly as free base and not as salt.

But the experimental results supporting this conclusion are not so numerous as with cresyl blue.[1] There is a greater possibility of injury to cells exposed to azure B than to cresyl blue. Since the rate of penetration at pH 9.2 is much slower with azure B than with cresyl blue, it was necessary to use a higher concentration[2] of azure B in order to maintain a comparable length of exposure to both these solutions. Cells were dead in this azure B solution after 3 hours' exposure while these cells in the cresyl blue solution were not dead until after 6 hours' exposure. Since injury hastens the entrance into the vacuole of substances whose penetration under normal conditions is negligible it is

[2] A lower concentration of azure B at pH 9.2 (causing penetration of 0.03 per cent dye in 15 minutes) was employed in which a rise in the pH value of the sap was found to be at about 0.5 pH over that of the control, and in which cells lived somewhat longer than in the solution described in the text. But even in this case, the results were not so conclusive as with cresyl blue.

all the more necessary to ascertain whether the cells were injured to such an extent that alkaline substances other than the azure B penetrated. Unfortunately it was not possible to determine this point so

TABLE I

pH Values of the Sap of Living Cells of Nitella flexilis. (A and B)

In presence of azure B and methylene blue. Average temperature 22.5°C. Duration of experiments was about 15 minutes with azure B and about 80 minutes with methylene blue (precaution was taken to avoid contamination of the sap). Electrode I was used unless otherwise stated.

	Conc. of dye in sap in per cent	pH of sap	Increase in pH of sap over the control sap	Conc. of dye in sap containing protoplasm in per cent	pH of sap containing protoplasm	Increase in pH of sap containing protoplasm over the control sap containing protoplasm
Nitella A, azure B						
Control	0	5.25		0	6.09	
Dye salt added *in vitro*	0.07	5.27	0.02	0.070	6.14	0.06
Free base of dye added *in vitro*	0.73	6.37	1.10	0.080	7.20	1.06
Penetration from 0.01 per cent dye at pH 9.2	0.075	6.39	1.14	0.070	6.73	0.64
Nitella B, azure B						
Control	0	5.52			6.00	
Penetration from 0.01 per cent dye at pH 9.2	0.08	6.52	1.0	0.080	6.50	0.5
Penetration from 0.01 per cent dye at pH 9.2. Electrode II	0.08	6.45	0.93	0.075	6.43	0.43
Nitella A, methylene blue						
Control	0	5.30		0	6.10	
Dye salt added *in vitro*	0.07	5.28		0.075	6.17	0.07
*Free base added *in vitro*	0.067	6.31	1.01	0.065	6.99	0.89
Penetration from 0.04 per cent dye at pH 9.2	0.07	6.43	1.13	0.073	6.78	0.61

* This free base is azure B extracted from methylene blue solution at pH 9.2.

satisfactorily as with cresyl blue. Owing to the heavy staining of the cell wall when cells were placed in the azure B solution (at all pH values) the dye at once began to diffuse into the vacuoles from the

cell wall when cells were transferred from the dye solution at pH 9.2, for example, into the buffer solution at the same pH value containing no dye so that it was not possible to prove whether or not the pH value of the sap would continue to increase without further penetration of the dye. Furthermore it was not possible to determine the penetration of azure B from tap water in the given period without employing a very high concentration of dye (too high for experiments without danger of causing injury to cells).

Thus the proof of the penetration of the dye in form of free base was less conclusive than with cresyl blue, but it seems probable that the azure B also penetrates as free base.

Sap Mixture[1] (*Sap Containing Protoplasm*).—Addition of 0.07 per cent dye salt or the free base to the mixture of sap and protoplasm produces much the same effect as on sap in that the dye salt brings about practically no alteration in the pH value of the mixture while the free base of the dye increases the pH value about 1 pH over that of the control mixture (Table I). But when the azure B penetrates until 0.07 per cent dye has collected in the vacuole, the increase in the pH value of this mixture is found to be only about 0.6 pH instead of 1 pH as was the case with the sap. Judging from the results obtained with penetration of dye from methylene blue (described in following section) we may conclude that this difference may be due to the production of acid by the protoplasm as a result of penetration of dye, and not to the penetration of azure B salt into the protoplasm. It is not due to the greater buffer action of the protoplasm since the pH value is increased to about the same extent when the azure B free base is added *in vitro* to the sap or to the mixture of the sap and the protoplasm. As the protoplasm consists of only a very thin layer it is not possible to obtain it free from the sap so as to study its behavior.

B. Nitella (B)

Approximately the same results were obtained when the experiments were repeated with *Nitella*[1] (*B*) (Table I).

On employing Electrode II, the same results were obtained (Table I).

Spectrophotometric measurements show that the dye which has penetrated the vacuole is like the free base dissolved in the sap *in vitro*, and the external dye solution employed in giving absorption

curves characteristic of azure B with primary absorption maxima at about 650 mμ.

III

Methylene Blue or Tetramethyl Thionin

Nitella (A)

The Sap.[1]—The pH value of the sap after 0.07 per cent dye had penetrated from 0.04 per cent methylene blue solution at pH 9.2 in 80 minutes was found to be about 1 pH higher than the control (Table I), which agreed closely with the rise in the pH value brought about by dissolving the 0.07 per cent free base of azure B in the sap (Table I). The methylene blue chloride when dissolved in the sap brought about practically no change in the pH value of the sap (Table I).

There was a striking similarity in the behavior of the dye absorbed by the vacuole and by the chloroform. When chloroform was shaken up with the methylene blue solution at pH 9.2, the dye appeared red in the chloroform. After evaporation of the chloroform, the dye residue was dissolved in sufficient sap to make the concentration 0.07 per cent. The pH value of this sap was found to be again about 1 pH higher than that of the control sap (Table I).

On determining by means of the spectrophotometric measurements the nature of the dye which has penetrated the vacuole from this methylene blue solution until 0.07 per cent has collected in the sap, the absorption curves showed that the dye consisted more of azure B than methylene blue with primary absorption maxima at 653 to 655 mμ. The dye absorbed by the chloroform was also found to be chiefly azure B with a primary absorption maximum at 650 mμ.

These results therefore confirm those obtained previously by spectrophotometric analysis[3,4] and support the conclusion previously made that the azure B free base present as impurity in methylene blue solution penetrates the vacuole so much more rapidly than methylene blue salt that the dye which collects in the vacuole consists chiefly of azure B and not methylene blue.

[3] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, **24**, 425; 1927–28, **25**, 563; *J. Gen. Physiol.*, 1928–29, **12**, 147, 407.

[4] Experiments on spectrophotometric measurements were repeated by Brooks, who found methylene blue to penetrate instead of azure B. Brooks, M. M., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 290. Protoplasma, 1929, **7**, 46.

As soon as cells are injured, however, the methylene blue penetrates more rapidly so as to shift the absorption curve of the dye in the sap from that of a mixture containing more azure B than methylene blue to that of a mixture containing more methylene blue than azure B. Since in the present instance where there is 0.07 per cent penetration of dye from methylene blue solution at pH 9.2 it is the azure B free base that still penetrates predominantly, the cells thus exposed cannot be injured to the extent of permitting the dye salts to penetrate freely although the cells are dead in the methylene blue solution within three hours' exposure.

Owing to this toxicity of methylene blue solution and to the necessity of longer exposure of these cells to the solution, the results are not so convincing as those obtained on cresyl blue, but the evidence is in favor of the conclusion that predominantly azure B in form of free base penetrates the vacuoles unless cells are injured.

"*Sap Mixture*"[1] (*Sap Containing Protoplasm*).—The same results as in the case of the sap were obtained when the methylene blue or the dye extracted by the chloroform from the methylene blue solution was dissolved in the sap mixture *in vitro* (Table I). But on penetration of the dye from the methylene blue solution at pH 9.2 into the vacuoles the pH value of the sap mixture was raised only 0.6 pH over that of the control sap mixture instead of 1 pH increase, as was the case of the sap. This difference is not due to the greater penetration of methylene blue into the protoplasm than into the sap, because the dye in the sap mixture gives the same absorption curve as the dye in the sap, with a primary absorption maximum at 653 to 655 mμ (characteristic of a mixture of azure B and methylene blue with preponderance of the former). In all probability it is due to the production of acid by the protoplasm as a result of the dye penetration.

IV

Crystal Violet

Nitella (A)

Crystal violet is more toxic than other dyes employed here and the rate of penetration at any pH value into the cells of *Nitella* is not sufficiently rapid to cause an adequate penetration of the dye before there is a danger of injury to cells. In 0.001 per cent dye solution, for ex-

ample, the cells are dead after 1 hour, while the penetration after 15 minutes is too small to show whether the dye increases the pH value. The rate of penetration is approximately the same at pH 9.2 as at pH 5.5. As the dye penetrates, a violet precipitate appears in the sap with very little of the dissolved dye. The penetration of the dye is in all probability mostly due to injury. Crystal violet is a basic dye and it is soluble in chloroform so that according to Overton's theory it should penetrate the cells rather rapidly. Since the penetration is found to be slow, it is of interest to find out more about the behavior of this dye toward the sap of *Nitella in vitro* as a basis for an explanation of why it does not penetrate the vacuole readily.

When 0.07 per cent crystal violet chloride was dissolved in the freshly extracted sap, the pH value was not higher than that of the control sap (about pH 5.3).

The dye was absorbed by chloroform from aqueous solution at pH 5.5 and pH 9.2 in about the same amount. The stained chloroform was freed from the aqueous solution, allowed to evaporate, and the colored residue was dissolved in the sap. This did not raise the pH value of the sap above that of the control sap (whether the dye thus dissolved was obtained by shaking the chloroform with crystal violet solution at pH 5.5 or pH 9.2).

The behavior of crystal violet in sap is therefore different from that of the free base of cresyl blue or of azure B in that with crystal violet there is no difference between the dye salt and the dye absorbed by the chloroform from the solution at pH 9.2, while with cresyl blue or azure B the dye salt does not alter the pH value of the sap while the dye absorbed by the chloroform from the dye solution at pH 9.2 increases it.

Spectrophotometric determination shows that the absorption curves characteristic of crystal violet are obtained with the dye in buffer solutions at pH 9.2 or at pH 5.5, or with the dye which was absorbed by the chloroform from these solutions.

V

Theoretical Considerations

Do these facts help us to understand why azure B enters the vacuoles of living cells of *Nitella* rapidly while methylene blue and crystal violet penetrate very slowly?

The results show that in aqueous solution azure B exists chiefly in two forms, the dye salt and free base, while methylene blue and crystal violet exist chiefly in one form. They also show that azure B penetrates chiefly as free base and not as salt. Since the free base of azure B predominates over the salt at higher pH values, the rate of penetration of the dye increases as the external pH value rises. The increase of free base over salt depends on the "apparent dissociation constant" of the dye.

As soon as the dye in form of free base passes through the protoplasm and comes in contact with the sap in the vacuole, the greater portion of it is converted into the dye salt. The extent of this conversion is dependent on the basicity of the dye, and on the constituents of the sap (such as the hydrogen ion, organic salts, and protein). With *Nitella* sap, the chief factor controlling the conversion is the pH value of the sap. Owing to the low pH value of the sap and to the apparent dissociation constant of azure B, the free base of the dye is converted to the salt to such an extent that only a very small proportion of the dye exists in the sap as free base. At equilibrium the concentration of free base of the azure B in the sap is proportional to that of the free base of the dye in the external solution, so that the higher the concentration of the free base outside, the higher is the concentration of the total dye (free base and salt) in the sap. This explains why at equilibrium the concentration of the total dye in the sap is found to be higher when the pH value of the external solution is raised or the concentration of the dye is increased.

Why is it that the free base of the dye penetrates more rapidly than the dye salt? In these cases the living cells of *Nitella* behave as if the rate of penetration of the dye into the vacuoles is controlled by three phases only, that is, the non-aqueous layer lying between the external aqueous phase and the internal aqueous sap in the vacuole. The rate of penetration is partly controlled by the partition coefficients of the dye at these two phase boundaries. Azure B free base, for example, diffuses into the non-aqueous layer readily because the partition coefficient K_e of this form of dye between the non-aqueous layer and the external solution is high. But no matter how high the K_e is, the dye cannot diffuse into the vacuole unless the partition coefficient K_i of the free base between the non-aqueous layer and the sap is

low or the free base is largely converted by the sap into another form, the K_i of which is low. With azure B, the free base is predominantly converted to the salt, the K_i of which is low, so as to promote a rapid penetration and accumulation of the dye in the sap. The rate of penetration of dye will be greatly reduced by increasing the pH value of the sap and so causing less transformation of free base to the salt.

The exit of the dye may also be accounted for on the same theoretical basis which explains why the rate of exit is hastened with a decrease in the external pH value.

Methylene blue does not penetrate the vacuole readily because it exists as salt even at a high pH value such as pH 9.2, and the partition coefficient K_e of the dye salt is low.

The reason crystal violet does not penetrate the vacuoles readily, though the partition coefficient K_e is high, is because the partition coefficient K_i of the dye is also high. The results indicate that crystal violet exists only in one form in the range of pH values available for living cells of *Nitella* (between pH 5 and pH 9.2), so that there is no such transformation of free base into the salt as shown by azure B. It is uncertain whether the crystal violet exists as salt or as free base. From the structure it appears to be a strongly basic dye, but owing to its solubility in lipoid it may be a very weak base. In case it is a weak base, it is too weak to increase the pH value even of the distilled water.

Owing to the penetration being dependent (at least in part) on more than one partition coefficient, the theory was called the "multiple partition coefficient theory."[5] It is uncertain yet whether this non-aqueous layer is lipoid in nature but its behavior is very much like that of lipoid. This theory is not identical with Overton's even if the non-aqueous layer proves to be a lipoid because Overton considered only one partition coefficient, K_e, between the non-aqueous layer and the external solution. His theory is tenable in so far as those dyes which are not soluble in lipoid do not readily penetrate the vacuoles of these living cells, but it fails to explain why those dyes which are soluble in a lipoid do not readily penetrate. In the case of crystal violet, for example, it becomes explainable only when the second par-

[5] For the theory of multiple partition coefficients see Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1927–28, **25**, 127; *J. Gen. Physiol.*, 1927–28, **11**, 111; 1928–29, **12**, 147, 407.

tition coefficient, K_i, is considered. Furthermore, the accumulation of the dye in the vacuole cannot be explained on the basis of his theory.

Such instances show the simplest type of penetration. In more complicated cases we must consider other partition coefficients. In view of the fact that a living cell of *Nitella* consists of a heterogeneous system composed of an outer non-aqueous layer (in contact with the external solution), the middle aqueous layer of the protoplasm, the inner, non-aqueous layer (in contact with the sap in the vacuole), and the aqueous sap, the rate of penetration may be controlled by two or more of the four partition coefficients. The discussion of these cases will be deferred to a later publication.

SUMMARY

Glass electrode measurements of the pH value of the sap of cells of *Nitella* show that azure B in the form of free base penetrates the vacuoles and raises the pH value of the sap to about the same degree as the free base of the dye added to the sap *in vitro*, but the dye salt dissolved in the sap does not alter the pH value of the sap. It is concluded that the dye penetrates the vacuoles chiefly in the form of free base and not as salt.

The dye from methylene blue solution containing azure B free base as impurity penetrates and accumulates in the vacuole. This dye must be azure B in the form of free base, since it raises the pH value of the sap to about the same extent as the free base of azure B dissolved in the sap *in vitro*. The dye absorbed by the chloroform from methylene blue solution behaves like the dye penetrating the vacuole. These results confirm those of spectrophotometric analysis previously published.

Crystal violet exists only in one form between pH 5 and pH 9.2, and does not alter the pH value of the sap at the concentrations used. It does not penetrate readily unless cells are injured.

A theory of "multiple partition coefficients" is described which explains the mechanism of the behavior of living cells to these dyes.

When the protoplasm is squeezed into the sap, the pH value of the mixture is higher than that of the pure sap. The behavior of such a mixture to the dye is very much like that of the sap except that with azure B and methylene blue the rise in the pH value of such a mixture

is not so pronounced as with sap when the dye penetrates into the vacuoles.

Spectrophotometric measurements show that the dye which penetrates from methylene blue solution has a primary absorption maximum at 653 to 655 mμ (i.e., is a mixture of azure B and methylene blue, with preponderance of azure B) whether we take the sap alone or the sap plus protoplasm.

These results confirm those previously obtained with spectrophotometric measurements.

A STUDY OF THE BACTERICIDAL ACTION OF ULTRA VIOLET LIGHT

III. The Absorption of Ultra Violet Light by Bacteria

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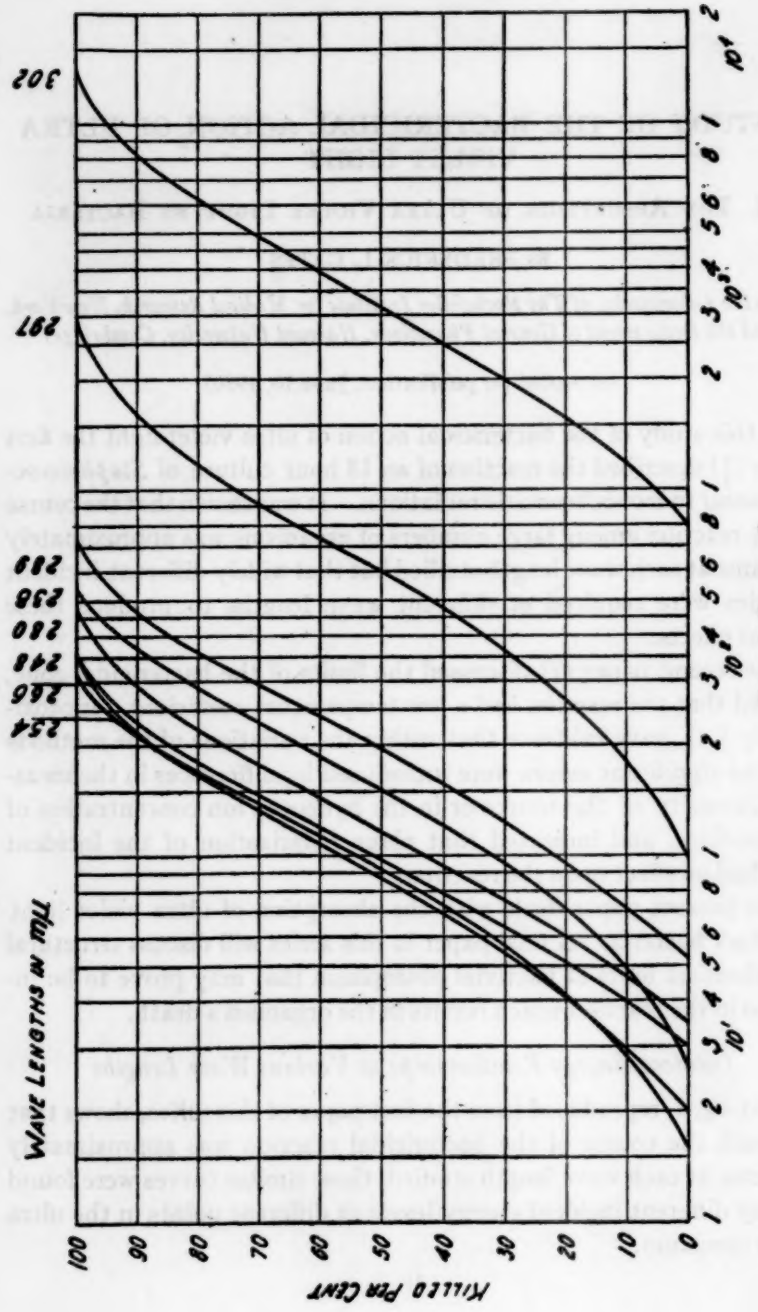
In this study of the bactericidal action of ultra violet light the first paper (1) described the reaction of an 18 hour culture of *Staphylococcus aureus* to monochromatic radiations. It was shown that the course of the reaction among large numbers of organisms was approximately the same at each wave length studied but that widely different incident energies were required at different wave lengths to produce these similar effects.

The second paper (2) discussed the limits of the bactericidal zone, showed that the reaction had a low temperature coefficient, (approximately 1.1), gave evidence that within the variations of the methods used no significant errors were introduced by differences in the measured intensity of the source or in the hydrogen ion concentration of the medium, and indicated that plane polarization of the incident light had no effect upon the reaction.

The present paper deals with the absorption of ultra violet light by intact bacteria. A final paper in this series will discuss structural and chemical units of bacterial protoplasm that may prove to be involved in the reaction which results in the organism's death.

Incident Energy Relationships at Various Wave Lengths

Text-fig. 1, reproduced from the first paper of this series, shows that although the course of the bactericidal reaction was approximately the same at each wave length studied, these similar curves were found at very different incident energy levels at different points in the ultra violet spectrum.



TEXT-FIG. 1. Incident energies required for bactericidal action at various wave lengths in the ultra violet spectrum

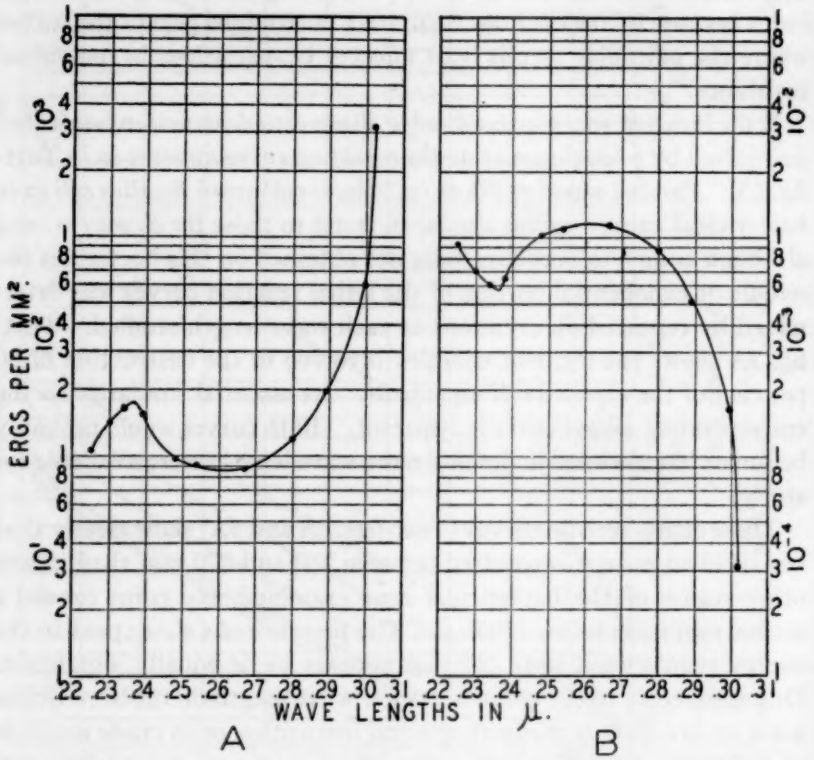
For example, if the incident energy required to kill half the exposed staphylococci be taken as an index, this energy requirement ranged from 3,150 ergs per mm.^{2} at 302 m.μ, a wave length near the limit of bactericidal action, to 88 ergs at 266 m.μ. At other wave lengths, either above or below 266 m.μ, more incident energy was required. The destruction of 50 per cent of the microorganisms is chosen as the index because it is in the most accurately determined part of the curves where the mortality rate is least affected by variations in individual resistance.

If the incident energies involved in 50 per cent destruction be plotted and joined by a continuous line the resulting curve appears as in Text-fig. 2A. Parallel experiments on an 18 hour culture of *Bacillus coli* gave bactericidal energy curves similar in trend to those for *S. aureus*, and although complete statistics were not obtained on this bacterium the middle or exponential portion of the lethal reaction curves was determined by repeated observations at each wave length studied. Text-fig. 3A shows the incident energies involved in the destruction of 50 per cent of the exposed *coli* organisms. Its essential similarity to the corresponding *aureus* curve is apparent. Both curves would probably be somewhat modified in detail if more wave lengths were available for study.

These characteristic curves (Text-figs. 2A and 3A) show clearly that less incident energy is required between 260 and 270 m.μ than in any other region of the bactericidal zone examined, and point toward a second minimum below 230 m.μ. The presence of a sharp peak in the energy requirement near 240 m.μ appears to be equally significant. Due apparently to the use of but a few wave lengths in the bactericidal zone, or to failure to measure spectral intensities, or to crude methods of estimating bacterial destruction, the occurrence of a minimum at about 266 m.μ, and of the peak in the curve near 240 m.μ has been overlooked by most investigators. Usually they have been content with the conclusion that the shorter the wave length the more marked the bactericidal action (3).

Bang (4) was apparently the first to observe regions of special bactericidal "effectiveness" or of corresponding bacterial susceptibility. Using a 30 ampere carbon arc through a spectrograph at 20 m.μ intervals, and estimating bactericidal efficiency mainly by relative exposure, he found two regions of maximal action, an

"inner maximum" between 340 and 360 m.μ, and an "outer maximum" between 240 and 260 m.μ. The lethal exposure varied from 1920 seconds at 330 to 300 m.μ through 120 seconds at 300 to 280 m.μ to 4 seconds at 280 to 260 m.μ and 2 seconds at 260 to 240 m.μ. Then longer exposures were required. The zone from 240 to 220 m.μ needed 20 seconds, that between 220 and 210 m.μ, 30 seconds, and that between 210 and 200 m.μ required 120 seconds exposure to kill the organisms.



TEXT-FIG. 2. A. Curve of incident energies involved in the destruction of 50 per cent of *S. aureus*.

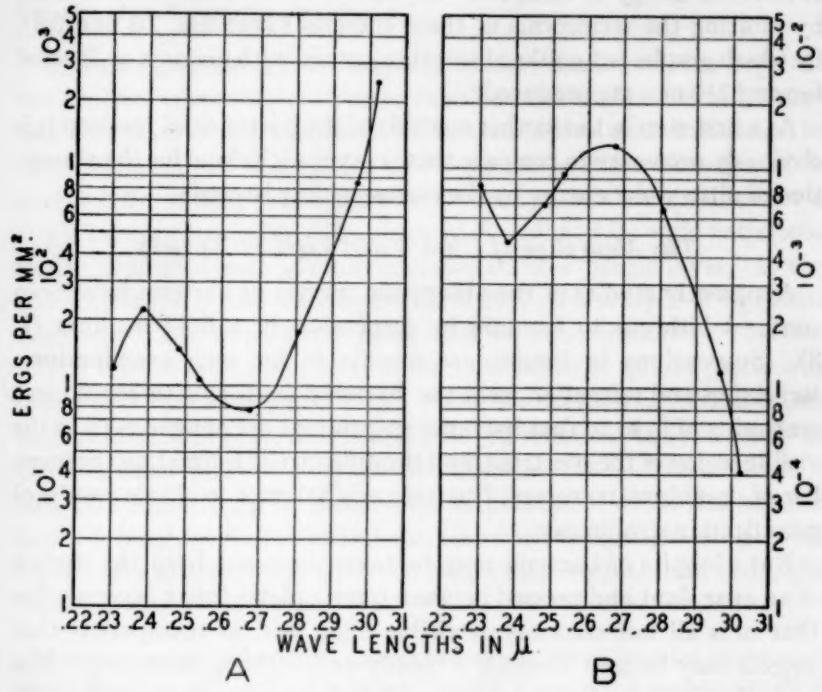
B. Curve of the reciprocals of 2A.

These longer exposures at short wave lengths were evidently due to the rapid decrease in intensity of the source employed. Mme. Henri (3) thought that both of Bang's maxima should be ascribed to variations in intensity of his carbon arc.

Newcomer (5) exposed *B. typhosus* in quartz capillary tubes to narrow bands of the iron arc spectrum and counted surviving bacteria plated in agar, after exposures of 5 or 10 minutes. His figures, like Bang's, indicate a region of maximum effect

between 254 and 268 m.μ, but his iron arc varied so much in spectral intensity that he did not find any significance in this peak of effectiveness. He concluded that "equal intensities produce equal effects in the regions 2100–2800. If there is a maximum in this region it is at most only slight and would be in the neighborhood of 2600."

When Mashimo (6) varied the exposure of bacteria in a spectrograph from 15 seconds to 80 minutes he found the first evidence of bactericidal action at 275 m.μ.



TEXT-FIG. 3. A. Curve of incident energies involved in the destruction of 50 per cent of *B. coli*.

B. Curve of the reciprocals of 3A.

With somewhat longer exposures the zone widened rapidly so that with a 3 minute exposure it extended from below 210 to above 280 m.μ. The marked action at 275 m.μ was evidently due to a relatively high intensity of his source at this wave length, as an examination of his published photograph shows.

Thus variations in spectral intensity, with no adequate methods of measurement or control, made it difficult to interpret the maxima found by Bang, Newcomer, and Mashimo, and these authors laid no stress upon them.

In the present study the measurement of the monochromatic radiant energy in absolute units focuses attention upon the marked differences in the incident energies required at different wave lengths to kill *S. aureus* and *B. coli*. The shape of the energy curves for 50 per cent destruction immediately suggests that it is the specific absorption of the ultra violet radiations that gives the curves significance. This relation of incident energy to its specific absorption is made the more striking by plotting the reciprocals of these energies (Text-figs. 2B and 3B), by which graphs not unlike absorption curves, with maxima at 266 and beyond 230 m.μ are produced.

As a first step in the further analysis of the bactericidal reaction it is obviously necessary to compare these curves with those for the absorption of ultra violet energy by the corresponding bacteria.

The Absorption of Ultra Violet Light by Bacteria

Apparently studies of the absorption spectra of bacteria have been confined hitherto to bacteria in suspension in a fluid medium (7, 8). Suspensions in liquids are unsuitable for such examinations. Reflection and refraction from the bacterial bodies, with consequent scattering of light so that bacterial suspensions are opaque even in the visible region of the spectrum, and the difficulty of estimating the number of organisms traversed, frustrate any attempt to obtain results of quantitative significance.

But a loopful of bacteria may be taken *en masse* from the surface of an agar slant and pressed between quartz plates into a layer so thin that is is all but colorless in visible light, and so transparent that objects may be seen through it clearly and without distortion. The bacteria are in optical contact and form a homogeneous medium for the transmission of visible or ultra violet light. Such a film is composed almost entirely of bacterial cells and the immediate products of their metabolism. Tests show that films of like thickness of the agar medium from which the bacteria were removed absorb no significant amounts of ultra violet light.

Such a film of bacteria may be set up in one optical path of a quartz photometer, with similar plates of quartz and a drop of glycerol in the other path as a control, and the absorption coefficient of the organisms readily obtained in the manner commonly employed for chemical solutions (9).

Two beams of light from the same source, which have passed through the specimen under examination or its control in the photometer, are spread by a quartz spectrograph into parallel spectra in the plane of a photographic plate. The energy that traverses the control path is subject to quantitative variation by means of a sector shutter, and with the shutter set at predetermined openings a series of photographs is made. Then the point, or points, of equal blackening in each pair of spectral photographs indicate the wave lengths at which the test specimen and the sector shutter in the control path have reduced the original intensity of the light to the same degree.*

In such experiments it is necessary to know accurately the depth of the medium traversed in order to calculate the coefficients of absorption for a layer of unit thickness. The standard or unit of thickness in these observations was chosen as 0.8 μ, the average diameter of *S. aureus* (10), so that the coefficients of absorption were obtained for a single layer of bacteria. The shape of *B. coli* and its wide variations in size precluded even so crude an estimate of a "single layer," so the coefficients for *B. coli* were figured arbitrarily for a layer of the same thickness (0.8 μ) to permit a comparison with the results for *S. aureus*.

The thickness of these films of bacteria between quartz plates was found to lie between 5 and 15 μ. Since the method of measurement employed (11) is easy, and is accurate (in microns) to the second or third decimal place it may be described in brief. The method is based on interferometry, namely, the measurement of the interference bands formed by the coincident spectra of white light reflected into a wavelength spectroscope from the two quartz surfaces enclosing the bacterial film.

A point source of white light (carbon arc, concentrated tungsten filament, or even a flashlight bulb with the filament vertical), is set up at a measured angle of incidence (60°) to the film specimen, so that its light is reflected from the back surface of the first quartz plate and from the front surface of the second (the surfaces enclosing the film), into a wavelength spectroscope. The reflecting surfaces must be chosen near the bacterial film, but not to include it, since adequate reflection occurs only when air is the medium between the two plates. With a proper set-up, and films of suitable thickness a series of vertical interference bands will cut across the spectrum.

* Measurements of absorption by such films made in 1923 with thermocouple and galvanometer (*Proc. Soc. Exp. Biol. and Med.*, 1923, **21**, 61), were evidently not as accurate as those obtained by the present method and have been discarded.

Then the distance (t) between the two plates (the thickness of the included film) is found by noting the wave lengths (λ_1 and λ_2) between which any convenient number (n) of interference bands is counted for substitution in the following equation:

$$t = \frac{n \lambda_1 \lambda_2}{2 \mu \cos r (\lambda_1 - \lambda_2)}$$

μ is the refractive index of the medium, and r is the angle of incidence and reflection. Since air is the medium $\mu = 1$, and with r at 60° $\cos r = 0.5$ so that the equation becomes

$$t = \frac{n \lambda_1 \lambda_2}{(\lambda_1 - \lambda_2)}$$

For example, with the apparatus set up as described 5 interference bands are counted between $\lambda_1 = 7241$ Ä u. and $\lambda_2 = 4638$ Ä u. It is only necessary to multiply these waves lengths together, divide by their difference, and multiply the result by 5 to determine that the distance between the plates *in the area examined* is 6.45 μ or 0.00645 mm.

In these experiments the films of bacteria, pressed out by hand, were not strictly plane parallel, but often thicker at one edge than at the opposite one, so four readings were taken at 90° intervals around the rim of each film and combined for an average thickness. The differences in thickness in the small central area exposed in the photometer were not so great as to make it desirable to obtain a mean exponentially according to Lambert's law. For example:

Film 1. *B. coli.* 90° readings at edge of film: 5.58, 6.17, 5.63, and 5.66 μ. Average 5.76 μ.

Film 2. *S. aureus.* 90° readings at edge of film: 9.21, 11.66, 11.24, and 10.53 μ. Average 10.66 μ.

Given the average thickness of each film, the number of layers (n) of bacteria it contained was then available for the determination of the coefficients of absorption for a layer 0.8 μ thick. For this purpose the familiar equation of Lambert's law was used, to which Wood (11) says that no exception has ever been found that was not attributable to experimental error. Thus if a is the fraction of the original intensity (I_o) transmitted by each unit layer, the intensity (I_t) observed after passage through n layers is given by the equation:

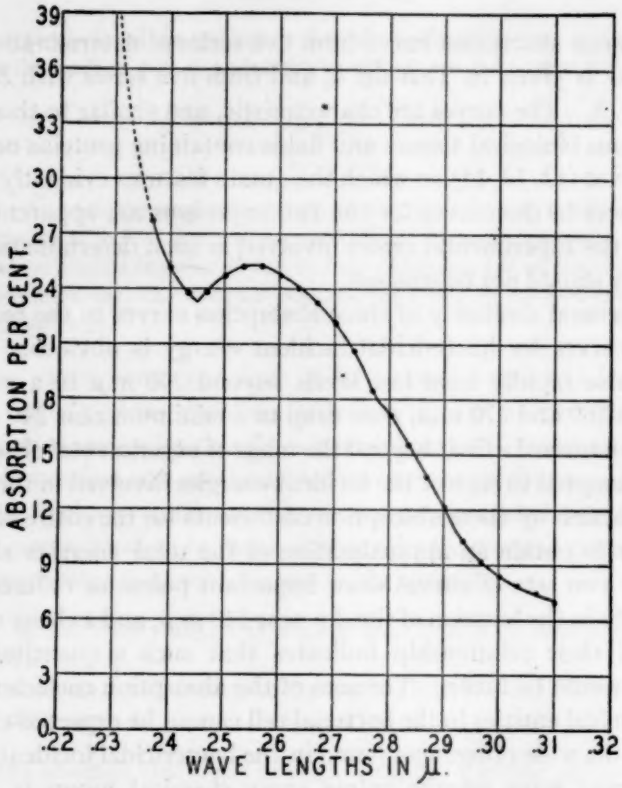
$$I_t = I_o a^n$$

The relation of I_t to I_o is chosen at appropriate intervals on the sector shutter of the quartz photometer, as described above, and this equation solved for a gives the coefficient of transmission at the wave length where the two spectra match. Then 1-a is the corresponding coefficient of absorption for a single layer of bacteria 0.8 μ thick.

The mean absorption curve from five series of determinations with *S. aureus* is given in Text-fig. 4, and from five series with *B. coli* in Text-fig. 5. The curves are characteristic, and similar to those found for various biological tissues and fluids containing proteins or protein derivatives (12, 13, 14) on which their main features evidently depend. Differences in the curves for the two organisms are apparent, but in view of the experimental errors involved in such determinations they probably should not be stressed.

The general similarity of these absorption curves to the reciprocals of the curves for bactericidal incident energy is obvious. All four curves rise rapidly from low levels beyond 300 m.μ to a maximum between 260 and 270 m.μ, then drop to a minimum near 240 m.μ and rise again toward a limit beyond the range of experimental observation. One is tempted to correct the incident energies involved in the bactericidal reaction by these absorption coefficients for the entire organisms in order to obtain an approximation of the total energies absorbed. Yet the two sets of curves show important points of difference also, especially in the location of the dip near 240 m.μ, and a closer consideration of their relationship indicates that such a quantitative correction would be futile. The sum of the absorption coefficients of all the chemical entities in the bacterial cell cannot be expected exactly to correct the wide differences found in the bactericidal incident energies at different wave lengths unless every chemical group is involved in the bactericidal reaction in exact proportion to its contribution to total absorption. Such a hypothesis is hardly tenable. It seems more probable that it is the effect of ultra violet energy on a single vital and sensitive structural or chemical unit that results in subsequent failure in cell multiplication. Rahn (15) has recently figured from the curves of abiotic reactions among large numbers of single cell organisms that death of the cell probably involves only a single chemical entity. As Coblentz and Fulton (16) have suggested, it is to be

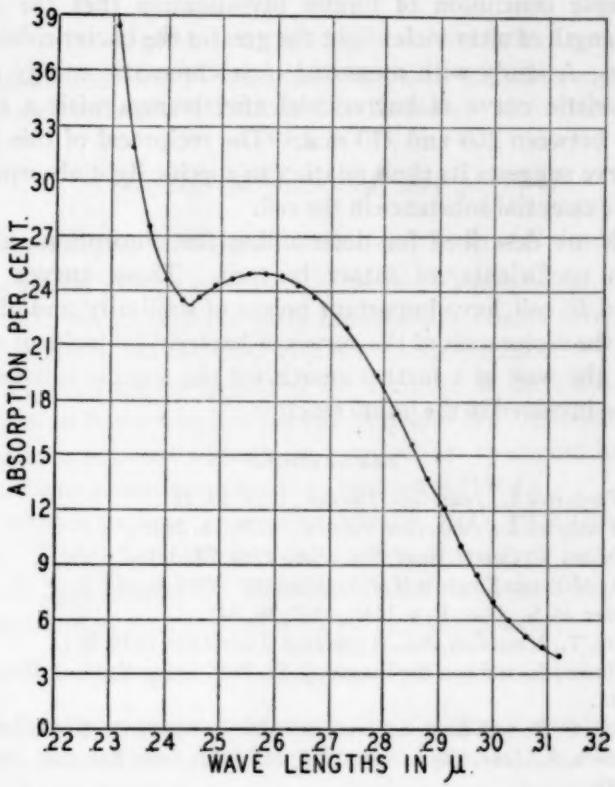
presumed that only a small fraction of the total absorbed energy first affects such an essential structure, and so leads to the death of the cell. Neither the reciprocals of the energy curves, which are undoubtedly modified by the absorption of light by elements not involved in the



TEXT-FIG. 4. The coefficients of absorption of ultra violet light by a layer of *S. aureus* 0.8 μ thick.

bactericidal reaction, nor the curves for total absorption give an accurate picture of the absorption curve of this vital substance, whatever it may be. Yet their similarities and their differences are alike useful in the further search for such an essential and sensitive element in the cell's structure and economy.

Thus it may be predicted that there are a number of chemical entities or aggregates in the living cell which have rather similar coefficients of ultra violet absorption, and that the sum of these similar absorption curves largely determines the shape of the curve for the entire cell.



TEXT-FIG. 5. The coefficients of absorption of ultra violet light by a layer of *B. coli* 0.8 μ thick.

Among these substances will be found the one essential element first affected by ultra violet light in the bactericidal reaction, and its absorption curve will be similar to, though not identical with, the reciprocal of the lethal energy curve. Finally it seems improbable that this sensitive substance is uniformly distributed throughout the cell's pro-

toplasm. An examination of the evidence for its concentration in the cell nucleus, and the further search for evidence of its chemical character are reserved for the final paper of this series.

SUMMARY

The simple conclusion of former investigators that the shorter the wave length of ultra violet light the greater the bactericidal action is in error. A study with measured monochromatic energy reveals a characteristic curve of bactericidal effectiveness with a striking maximum between 260 and 270 m.μ. The reciprocal of this abiotic energy curve suggests its close relation to specific light absorption by some single essential substance in the cell.

Methods are described for determining the absorption curve, or absorption coefficients, of intact bacteria. These curves for *S. aureus* and *B. coli* have important points of similarity and of difference with the reciprocals of the curves of bactericidal incident energy, and point the way in a further search for the specific substance, or substances, involved in the lethal reaction.

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THE REACTIONS OF CYANIDE WITH GLOBIN HEMOCHROMOGEN

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Hemochromogen and the Cyanide Compounds of Reduced Heme

Four pyrrol groups can combine to form a porphyrin. There are many different porphyrins with different side chains (Fischer, 1927). Each porphyrin can combine with iron to form a heme. The familiar hemin crystals are composed of the chloride of the particular iron-porphyrin or heme which is part of hemoglobin. Hemochromogen is defined as a pigment containing heme whose spectrum has the two characteristic bands first described by Stokes (1864).

In previous papers (Anson and Mirsky, 1925, 1928) it was shown that (1) Every hemochromogen consists of reduced heme joined to some nitrogen group; (2) Hemochromogen is always partly dissociated into and in equilibrium with its two components:

$$\text{Hemochromogen} \rightleftarrows \text{Reduced heme} + \text{Nitrogenous substance};$$

(3) Ordinarily, nitrogen substances react with reduced heme to form only one hemochromogen-like pigment. Cyanide, however, can react with reduced heme to form two different hemochromogen-like pigments; (4) The first cyanide compound of reduced heme contains one cyanide group per heme.

These results have been confirmed and evidence further given that the second cyanide compound contains two cyanide groups per heme (Hill, 1929).

The properties of cyanide are such as to permit of the estimation of the composition of the two cyanide compounds of reduced heme. But

it has not been clear what the relation is of these two compounds, both of which resemble hemochromogen spectroscopically, to an ordinary hemochromogen such as globin hemochromogen. The nitrogenous substances other than cyanide have the advantage of forming with heme only a single hemochromogen-like pigment which is undoubtedly a typical hemochromogen. But the properties of the other nitrogenous substances, as will be explained later in this paper, are such as to make difficult and uncertain the estimation of the compositions of the hemochromogens which they form from heme. As a result it has not been known whether hemochromogen in general contains one or two nitrogen groups per heme and it has not been known which of the two cyanide compounds is the typical hemochromogen, or why cyanide in particular forms two compounds with reduced heme. It was stated in the previous paper (Anson and Mirsky, 1928) that the relation of the cyanide compounds to ordinary hemochromogen was being studied by an investigation of the reactions of cyanide with heme in the presence of other nitrogenous substances.

The present experiments show that cyanide can react with globin hemochromogen in two entirely different ways. First, cyanide without displacing globin can combine with globin hemochromogen. In this reaction cyanide is *not* competing with globin for a place of attachment to the heme; it is behaving not like a typical nitrogenous hemochromogen-forming substance but probably like carbon monoxide. In addition, however, cyanide can displace globin from its combination with heme just as can ammonia and other nitrogenous substances (Anson and Mirsky, 1925, 1928). In the light of these results, the simplest although not the only explanation of the reactions of cyanide with heme in the absence of globin is that cyanide first combines with heme to form a typical hemochromogen containing one nitrogen group per heme and then combines with this cyan-hemochromogen just as it combines with globin hemochromogen.

The Biological Importance of the Hemochromogens

The biological interest in the chemistry of hemochromogen used to consist solely in its relation to the chemistry of hemoglobin which was the only heme pigment of known physiological importance. It is now clear that hemochromogens as such are of wide distribution and peculiar importance in nature. Pigments related to hemochromogen are present in aerobic tissues of plants and animals

generally in concentrations great enough to permit of direct spectroscopic observation (MacMunn, 1886; Keilin, 1925, 1926; reviewed in Anson and Mirsky, 1930b). In addition the investigations of Warburg and his colleagues show that there is universally present in much smaller amount another substance related to heme and probably to hemochromogen which plays an essential rôle in the catalysis of biological oxidations (reviewed in Warburg and Negelein, 1929, and Anson and Mirsky, 1930b).

The study of the heme pigments in nature has depended entirely on a knowledge of the chemistry of pure known heme compounds *in vitro*. It is therefore important to know as much as possible about the kinds of reactions in which heme pigments can take part. In particular the ability of heme to react with nitrogen groups is significant. Aerobic cells contain a substance which can catalyze oxidations. The catalysis stops when this respiratory enzyme is combined with carbon monoxide and goes on again when the carbon monoxide is dissociated from its compound with the enzyme by light (Warburg, 1926). Similarly heme can catalyze oxidations *in vitro* and it forms with carbon monoxide a catalytically inactive compound which can be dissociated by light. But the catalytic activity of the heme so far tried is much less than that of the cellular enzyme and the sensitivity of the heme's carbon monoxide compound to light is also much less. When heme is combined with some nitrogenous hemochromogen-forming nitrogen group then the catalytic activity of heme and the sensitivity of its carbon monoxide compound to light are very much increased (Krebs, 1928). In other words, combination with a nitrogen group makes heme more like the cellular respiratory enzyme (reviewed in Warburg and Negelein, 1929, and Anson and Mirsky, 1930b).

The Reactions of Globin and Cyanide with Heme

In the present experiments different amounts of cyanide are added to reduced heme in the presence of different concentrations of globin. From the spectra of the resulting solutions it can be decided when the cyanide is competing with globin for a place on the heme molecule and when the cyanide is not competing with globin. Before describing the experiments in detail it is necessary to recall what has already been shown to happen when either globin or cyanide alone is added to reduced heme.

If denatured globin is added gradually to an alkaline solution of reduced heme the spectrum of reduced heme is gradually and finally completely replaced by that of globin hemochromogen. The addition of further globin then causes no further change in the spectrum (Anson and Mirsky, 1928). If cyanide, however, is gradually added to reduced heme there appears first a typical hemochromogen spectrum whose α band is 50 Å to the blue of that of globin hemochromogen.

But then on further addition of cyanide there appears a second two-banded spectrum similar to but definitely different from that of a typical hemochromogen. The α band is 100 Å to the *red* of that of globin hemochromogen and is much weaker than the α band of either globin hemochromogen or the first cyanide compound. This second cyanide compound obtained by adding an excess of cyanide has long been known (see spectrum in Oppenheimer's Handbuch, 1909). The first cyanide compound, which can exist only in a narrow range of low cyanide concentrations, was not observed until recently (Anson and Mirsky, 1928).

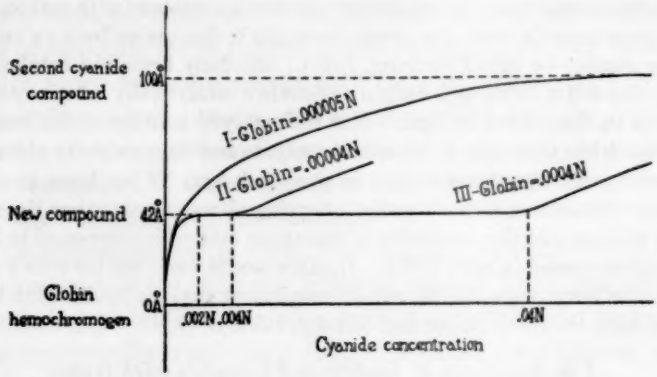


FIG. 1. Positions of α bands of pigments obtained on adding cyanide to globin hemochromogen.

The Reactions of Cyanide with Globin Hemochromogen

The new experiments are these:

1. To a dilute alkaline solution of globin hemochromogen prepared from hemoglobin (0.0084 per cent or 0.000005 N in respect to the iron of hemoglobin) there is added more and more cyanide. The α band is shifted more and more to the red, until after a shift of 100 Å it has reached the position characteristic of the second cyanide compound of reduced heme prepared by adding cyanide to heme in the absence of globin. (See Curve 1. In order to give a simple picture of all the phenomena in one graph, the graph has been made schematic and grossly inaccurate. The actual observations are given in the tables.)

2. More and more cyanide is added to globin hemochromogen in the presence of extra globin, the total globin concentration being 0.00004 N instead of 0.000005 N. The band moves to the red until when the cyanide concentration is 0.002 N, the band is 42 Å to the red of the band of globin hemochromogen. Doubling the cyanide concentration

TABLE I

CN / N	Position of α band − Å to red of 5585 Å		
	Globin = 0.0004 N	Globin = 0.00004 N	Globin = 0.000005 N
0	0	0	0
0.0001	18	18	16
0.0002	27	28	26
0.0004	33	34	33
0.001	39	40	44
0.002	41	42	49
0.004	43	43	
0.01		48	
0.02	42	57	

TABLE II

CN / Globin	Position of α band − Å to red of 5585 Å		
	Globin = 0.00008 N	Globin = 0.00004 N	Globin = 0.00002 N
0	0	0	0
125	45	43	
250	48	48	48
500	57	57	57
1,000	69	66	65
50,000			100
			No globin
			100

(making it 0.004 N) now causes no further change in the spectrum. But the addition of still more cyanide causes the band to move to the red again. (See Curve II.)

3. Finally, more and more cyanide is added to globin hemochromogen in the presence of a concentration of globin ten times greater still, namely 0.0004 N. Until the cyanide concentration is 0.004 N the

results are *precisely* the same as with the solutions containing ten times less globin. But whereas in the more dilute globin solution further addition of cyanide causes the band to shift to the red, in the solution 0.0004 N in respect to globin the cyanide concentration must be increased from 0.004 N to 0.04 N before the band is shifted to the red again. (See Curve III.)

4. Whenever the globin concentration is *greater* than 0.00004 N, the cyanide concentration is *less* than 0.002 N, and the band is accordingly *less* than 42 Å to the red of the band of globin hemochromogen, the position of the band depends solely on the cyanide concentration. Increasing the globin concentration has no effect on the spectrum so long as the cyanide concentration is kept constant.

5. Whenever the cyanide concentration is *greater* than 0.002 N and the band is *more* than 42 Å to the red of the band of globin hemochromogen, the position of the band depends solely on the *ratio* of the concentrations of cyanide and globin. Increasing the cyanide concentration has no effect on the spectrum provided only that the globin concentration is increased to the same extent.

6. The nature of the mixed spectrum given by a solution containing two different pigments depends on how far apart are the bands of the pigments. If the bands are far enough apart they do not fuse completely but can be distinguished separately in the mixed spectrum. For instance, the α bands of the first and second cyanide compounds of reduced heme which are 150 Å apart can both be seen in the spectrum given by a mixture of the two cyanide compounds (Anson and Mirsky, 1928). If the two bands of the two pigments are close enough together, however, they fuse to give a single band whose maximum absorption is at some wave length intermediate between the wave lengths at which the two individual pigments have their maximum absorptions. The more of one of the pigments is present the closer will the position of the intermediate band be to the band of that pigment. Thus the α bands of oxy and carbon monoxide hemoglobins (Hartridge, 1912) and of globin and ammonia hemochromogens (Anson and Mirsky, 1925, 1928) fuse to give intermediate bands whose positions are determined by the compositions of the mixtures. Finally, the bands of two pigments may not be close enough to mix to give a single intermediate band and still not far enough apart for two points of maximum

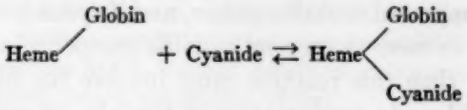
absorption to be distinguished easily in the mixed spectrum. In such cases the mixed spectrum has a band much broader than the band of a single pigment and this band becomes more and more asymmetrical as the relative concentration of one of the pigments in the mixture is increased.

In general the same mixed spectrum which is seen when one looks through a single cell containing a solution of two different pigments is also seen when one looks at the same time through two cells, one containing a solution of one pigment alone, the other containing a solution of the other pigment alone.

If one looks through two cells, one containing a solution of globin hemochromogen, the other a solution of the second cyanide compound of reduced heme, one sees a very broad asymmetrical band. It is impossible by means of optical mixtures of the spectra of globin hemochromogen and the second cyanide compound to imitate the single bands given by solutions of cyanide plus globin hemochromogen. All the spectra actually observed, however, can be imitated by means of optical mixtures of the spectra of globin hemochromogen, the second cyanide compound and the product with the band 42 Å to the red of the band of globin hemochromogen.

Two conclusions are drawn from the new facts:

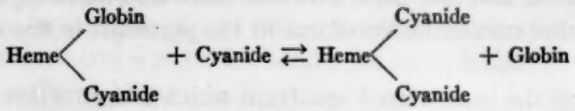
1. Cyanide can combine with globin hemochromogen without competing with or displacing globin to form a pigment with an α band 42 Å to the red of the band of globin hemochromogen.[1] In this reaction it is not behaving as a typical nitrogenous hemochromogen-forming substance but probably like carbon monoxide.



2. Cyanide can further react with cyanide-globin hemochromogen to form the second cyanide compound of reduced heme which contains

[1] Hill (1929) has observed an analogous pigment obtained by adding both nicotine and cyanide to heme. He believed that every hemochromogen contains two nitrogen groups per heme and that the compound of nicotine and cyanide with heme is a typical hemochromogen which happens to contain two different nitrogen groups.

two cyanide groups per heme. In this reaction cyanide is competing with and displacing the typical nitrogenous substance, globin.



From Curve I alone one might suppose that cyanide in reacting with globin hemochromogen simply displaces the globin and forms the second cyanide compound of reduced heme. On this basis any intermediate α band observed must be the result of the fusion of the α bands of globin hemochromogen and the second cyanide compound. This theory is disproved by the fact that one cannot imitate the observed spectra by means of optical mixtures of the spectra of globin hemochromogen and the second cyanide compound. To explain the mere existence of single intermediate bands one must assume the existence of a new compound with a band between the bands of globin hemochromogen and the second cyanide compound. Since the compounds of heme with either globin or cyanide alone do not have such a band, the new compound probably contains both globin and cyanide.

Curve III shows that cyanide first reacts with globin hemochromogen to form a new compound with a band 42 Å to the red of the band of globin hemochromogen and only then reacts further with this new compound. The extent of formation of the new compound is independent of the globin concentration, so globin is not being displaced. The new compound therefore contains globin as well as cyanide, a conclusion already drawn from Curve I.

If the new compound contains globin, and if cyanide reacts with the new compound to form the second cyanide compound which does not contain globin, then this reaction must involve the displacement of globin by cyanide. In confirmation of this the extent to which the second cyanide compound is formed from the new compound of cyanide and globin hemochromogen depends solely on the cyanide to globin ratio, which is precisely what one would expect were the cyanide and globin competing for the heme.

The reason why Curve I deviates from Curves II and III even before the cyanide concentration reaches 0.002 N is now clear. If the globin concentration is low enough some globin is displaced by cyanide even

if the cyanide concentration is not high enough to convert globin hemochromogen completely into cyanide-globin hemochromogen. To separate the two reactions the globin concentration must be above a certain minimum. Theoretically the two reactions can never be separated completely.

The fact that cyanide can displace globin from its combination with heme is most simply explained in the assumption that cyanide can react like a typical nitrogenous substance such as globin. This assumption, however, is not a necessary one. The affinity of hemoglobin for carbon monoxide depends on the hydrogen ion concentration; carbon monoxide and hydrogen ions compete for the hemoglobin and can displace each other. Yet these two groups do not react with hemoglobin in the same way. In all probability, carbon monoxide combines with the iron and the hydrogen ion does not, but combines with a group close to the iron and so influences the affinity of the iron for carbon monoxide.

Experimental Procedure

It remains to describe in detail the manner in which the values put down in the tables were obtained. In all cases the solutions were at O°C. in a cold room, the final concentration of NaOH was 0.5 N, solid sodium hydrosulfite ($Na_2S_2O_4$) was added as a reducer, and the bands read after 5 minutes with a reversion spetroscope (Hartridge, 1912). In some cases the bands were read again after several hours. No change was found. The purpose of the strong alkali was to bring out the hemochromogen-forming capacity of globin (Anson and Mirsky, 1928).

The three solutions of Table I were 0.000005 N, 0.00004 N and 0.0004 N in respect to globin. The 0.000005 N solution was prepared by adding 2.5 ml. of 0.2 N HCl to 1 ml. of 8.5 per cent horse carbon monoxide hemoglobin and then diluting with NaOH. This solution was accordingly 0.000005 N in respect to globin, heme and iron. The 0.00004 N and 0.0004 N solutions contained 0.00001 N globin and heme from hemoglobin and in addition denatured globin prepared by the oxalic acid-acetone method (Anson and Mirsky, 1930a).

The 0.00008 N solution of Table II contained 0.00002 N globin and heme from hemoglobin and in addition 0.00004 N globin prepared by the acetone method. To obtain the 0.00004 N and 0.00002 N solutions, the 0.00008 N solution was diluted with 0.5 N NaOH.

The band 42 Å to the red of the α band of globin hemochromogen is a sharp band intermediate in intensity as well as in position between the α bands of globin hemochromogen and the second cyanide compound of reduced heme.

When the cyanide to globin ratio reaches 1,000 the band begins to be too asymmetrical to be read with the reversion spectroscope.

Alkaline solutions of hydrosulfite attack the cements which were tried, including de Khotinsky cement. The decomposition products of the cements influence the spectra. This difficulty was avoided by using cells made of paraffin blocks and pieces of glass microscope slides. Such cells can be made in a few minutes. From paraffin blocks 1.5 cm. or 3 cm. thick depressions are melted out with a hot iron. These depressions are closed on the sides of the blocks by melting in pieces of glass. To make a double cell to permit looking through two solutions at once, a piece of glass which is to act as a partition is first melted between two blocks of paraffin. If cells only a few millimeters thick are desired, it is better to place slightly softened but not melted paraffin between two pieces of glass which are then pressed together in a vise until they are the desired distance apart. The glass sides of the cell are thus automatically made parallel. Finally some of the paraffin is melted out to form the cell.

The Nature of the Two Cyanide Compounds of Reduced Heme

A priori there are four possibilities in regard to the nature of the two cyanide compounds of reduced heme. Neither of the two cyanide groups may be a typical nitrogenous hemochromogen-forming group; both may be; cyanide may be acting as a typical nitrogenous group in forming only the first; or in forming only the second compound. The present experiments do not decide conclusively which of these four possibilities corresponds to the truth. What has been made clear is that cyanide can combine with heme in two different ways. In one reaction it does not compete with or displace globin; in the other it does. In the light of these results and of the fact that the spectrum of the first compound is similar to that of globin hemochromogen and the spectrum of the second compound is similar to that of cyanide globin hemochromogen, the simplest theory is that the first compound is a typical hemochromogen and the second compound a compound of cyanide with cyanide hemochromogen.

The Composition of Hemochromogen

If the first cyanide compound of reduced heme which has one cyanide group per heme is a typical hemochromogen, then it is probable that hemochromogens in general contain one nitrogen group per heme. It would obviously be desirable to confirm or refute this conclusion by independent investigation of the composition of some hemochromogen whose nitrogenous substance forms only one hemochromogen-like compound with heme. The attempts to do this have not yet led to any conclusive results. Von Zeynek found by analyses of

the precipitates that ammonia hemochromogen (1898) contains one nitrogenous group and pyridine hemochromogen (1910) two nitrogenous groups per heme. But such direct analyses must remain of dubious significance until it is shown that in the preparation of the precipitates no hemochromogen-forming nitrogen groups are split off from the heme and no non-hemochromogen-forming nitrogen groups added to the carboxyl groups.

Two molecules of pyridine must be added to each molecule of heme in order to convert heme into pyridine hemochromogen (R. Hill, 1926, 1929). If all the pyridine added were combined with the heme this would mean that pyridine hemochromogen contains two pyridines per heme. But some of the pyridine added must be free to drive the equilibrium:

$$\text{Pyridine hemochromogen} \rightleftarrows \text{Heme} + \text{Pyridine}$$

to the left. It cannot be decided from Hill's experiments whether or not this free pyridine is negligible. In dilute solution of the pigment the concentration of free pyridine needed to prevent visible dissociation of the hemochromogen may be determined. It is not known whether the same concentration of free pyridine is needed to prevent visible dissociation of the hemochromogen in the concentrated solutions actually used. As has already been pointed out (Mirsky and Anson, 1929) the equilibrium is complicated by the aggregation and precipitation of both pyridine hemochromogen and reduced heme:

$$\begin{array}{ccc} \text{Pyridine hemochromogen} & \rightleftarrows & \text{Reduced heme} + \text{Pyridine} \\ \downarrow & & \downarrow \end{array}$$

Precipitation or aggregation of the hemochromogen would drive the equilibrium to the left and so reduce the concentration of free pyridine needed to prevent detectable dissociation of the hemochromogen. Precipitation of the heme would have the opposite result. It is not known what effect concentration has on the relative velocities and extents of the aggregation of the two pigments.

Dr. Northrop has suggested that it might be possible to estimate the free pyridine in a solution of pyridine hemochromogen by shaking the aqueous solution with a solvent which does not mix with water but in which pyridine is soluble, estimating the total pyridine in this solvent, and then calculating the free pyridine in the water from the distribution constant.

Cyanide and Carbylamine

Warburg, Negelein and Christian (1929) have recently described some reactions of carbylamine with hemoglobin which in some ways resemble the reactions of cyanide with heme. Carbylamine can combine with hemoglobin in two different ways. In one reaction it competes with carbon monoxide, in the other it does not. Native globin in hemoglobin is not behaving like a typical nitrogenous substance such as ammonia or denatured globin. Whether one of the carbylamines is behaving like a typical nitrogenous substance has not yet been determined.

Conclusions

Cyanide can react with globin hemochromogen in two different ways. In the first reaction cyanide combines with globin hemochromogen without displacing or competing with globin. In the second reaction cyanide displaces globin.

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THE EFFECT OF LOW PRESSURES ON CELL OXIDATION

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INTRODUCTION

It has long been a matter of common knowledge that not only human beings but most other animals as well as plants can endure great variations in their supply of oxygen without obvious ill effects. This is demonstrated by the existence of life at high altitudes, the phenomena of anaerobiosis and many other facts. In addition to the fundamental researches of Harden, Hill, Meyerhof, Warburg, and many others, which have demonstrated the difference between, and yet the interdependence of aerobic and anaerobic respiration, numerous attempts have been made to correlate the amount of oxygen present with the degree of activity of an organism. One of the most recent of these is that of Amberson (1928) who shows quite definitely that in the case of *Paramoecium* and fertilized *Arbacia* eggs the cell respiration continues at the normal rate, almost independent of the oxygen supply until the latter reaches an exceedingly low value (about 10 mm. Hg). Warburg (1926) also noticed the same phenomenon in the case of yeast. Most other investigators' results have agreed with these findings, among them those of Henze (1910), Lund (1918), Hamburger and St. Györgi (1925), Pütter (1924), Harvey (1925). The activities which were observed in this connection covered a wide range, including the actual uptake of oxygen itself (Amberson, Warburg). Mention may also be made of investigations concerning the effect of lowered oxygen tension on mammals (see Barcroft's book, 1925). There seems to be little doubt, therefore, that within wide but varying limits the organism is independent of the oxygen supply.

The above considerations apply to changes in oxygen tension where the tension is varied by taking different proportions of oxygen and nitrogen but where the total pressure of the system is held constant

at approximately 760 mm. Hg. Obviously the tension may also be varied by altering the *total pressure* where the relative gas tensions are held constant. This occurs both naturally and artificially when air is used and the barometric pressure is reduced (*e.g.*, high altitudes or low pressure chambers). The classic work of Bert (1878) shows the diversity of effects which might be obtained by a lowering (or raising) of the barometric pressure. More recently interest in this connection has been centered on the influence of these conditions on human reactions, an interest which, of course, has been stimulated by the war and the increasing importance of aviation.[1] Although the response of large animals to lowered pressures has been thoroughly studied very little attention has been paid to the effect of low pressures on the respiration of the individual cells and tissues themselves. The assumption has been tacitly made that the effect of a reduced oxygen tension whether secured by reducing the percentage of oxygen at atmospheric pressure, or by reducing the total pressure, would be the same. From the purely physical point of view such an attitude is very natural.

It seemed worth while, however, to test the validity of this assumption by a series of experiments in which the respiration of a simple organism would be measured under varying oxygen tension, these variations to be secured by altering *both* the percentage composition of the gases *and* the total pressure. The results of these experiments show quite clearly that there is a distinct and in some cases decided difference in the effect of gas tension depending on how the changes in tension are made. Or, in other words, it may be said that a reduced pressure, has, *per se*, an effect on tissue oxidation which is not obtained with a reduction of simply the oxygen tension.

Methods

The organism used in this work was baker's yeast, a fresh suspension of which was made up for each experiment in Ringer solution without bicarbonate, but containing 1 per cent glucose. The gas exchange was measured by means of a modification of the Warburg manometric method. The simple manometer, as well as the differential mano-

[1] J. Barcroft in his book, "The Respiratory Function of the Blood" (1925) and E. C. Schneider in a series of papers in the *American Journal of Physiology* have summarized what has been accomplished in this field.

meter, have been described fully in Warburg's book (1926), together with details regarding the shaking device and temperature regulation. The only modification of the method was made when it became necessary to measure oxygen absorption or carbon dioxide evolution under reduced barometric pressure.

For this purpose the simple manometer is clearly not adapted, since it is open on one limb. It is also impossible to use the differential manometer in its usual form because both limbs, or the containers attached thereto, must be initially at the same pressure. Therefore a special differential manometer was constructed. The manometer proper was of the usual type, very similar to illustration on page 1 of Warburg's book ("Über den Stoffwechsel der Tumoren, 1926"), with posterior extensions carrying two cups or vessels. Each of these was of the "Kegelgefäss" or "conical vessel" type with a side extension and a small insert of glass on the center of floor of the vessel for carrying alkali. Each of the side extensions was fitted with a ground-in glass stopper. All joints were carefully ground and greased and their resistance to leakage carefully checked. On the upward extension of each manometer limb there was a glass stopcock, *A* and *B*. Then the two extensions were joined by fused glass tubing in the form of an inverted *Y*, at the top of which was a three-way stopcock, *C*. Since the system was all of glass the only leaks could occur at the stopcocks. These were accordingly specially ground in, greased, and tested frequently against leakage.

The fluid used in the manometer was Brodie's solution. In order to provide for the introduction of the fluid it was necessary to open the bottom of the manometer and fuse on a piece of tubing with another stopcock. When the latter was opened the fluid could be run in to the desired level from below and the stopcock closed. The manometer then functioned in the usual manner. The glass part was fastened securely to a wooden back and carried on the shaker as described by Warburg. At the beginning of an experiment the desired amount of yeast suspension was placed in one of the vessels and an equal amount of Ringer solution in the other together with any other material needed. The vessels were then placed on the manometer and held in place by springs. If a special gas mixture was to be investigated, all the upper stopcocks (*A*, *B*, and *C*) were opened and the small stoppers on the side extensions of the vessels were removed. The gas was then allowed to flow through the entire apparatus from a tank or other source of supply as described by Warburg (1926, p. 3). Stopcock *C* was then closed and the small stoppers of the vessels replaced. This isolated the system *as a whole* from the outside air. But the two vessels remained in communication with each other through the arms of the inverted *Y*. The vessels were then placed in the water bath at 37°C. and shaken till temperature equilibrium had been attained. At this point stopcock *C* was opened for an instant to relieve the positive pressure generated by the heating of the gas inside the system. Then stopcocks *A* and *B* were closed. This cut off the communication at the top between the two vessels and a differential alteration of pres-

sure could then be observed on the manometer scale as in the case of any differential manometer.

Suppose now after taking several readings it was desired to reduce the pressure within the system. The stopcocks *A* and *B* were carefully opened thus equalizing the pressure in the two vessels. A heavy rubber pressure tube was attached to the outlet above stopcock *C* and a slight negative pressure put on. Cock *C* was then opened and the pressure reduced to any predetermined amount. Then cock *C* was closed and the same method used as under atmospheric pressure. If the pressure was very low it was usually necessary to repeat this procedure two or three times because after reduction the escape of gases from solution might alter the tension of the gas space of the vessels.

To obtain reduced pressures an oil vacuum pump was attached to a 10 gallon carboy. A line ran from the latter to the heavy tubing which could be attached to the manometer. In this line was included a mercury manometer, scaled in millimeters. Several heavy glass stopcocks variously placed allowed for cutting off the line at any desired point. All joints were paraffined and shellacked. In fact the system would hold a high vacuum without appreciable change for weeks. The carboy was exhausted as highly as the pump would permit. This insured as low pressures as were ever needed. After the stopcocks in front of and behind the carboy had been closed the carboy served as a reservoir of low pressure to be drawn on when desired.

For experiments with carbon monoxide it was necessary to generate the gas. This was done by the method described in Treadwell's "Analytical Chemistry" (11th German edition, 1923, vol. 2, p. 669), in which formic acid is run slowly into hot concentrated H_2SO_4. The gas formed was run through a condenser and flasks of water and soda lime, to remove water vapor and carbon dioxide. It was then mixed in desired preparations with oxygen in a gasometer and thence run through the manometer in the usual way.

The results may be expressed either on an absolute or relative basis. If the latter, the normal respiration may be called 100 per cent and any deviation expressed as a per cent of this normal. This method is often most convenient for comparative data in which the absolute quantities involved are of secondary consequence. However, for purposes of calculation the absolute amounts must be known. In this case we may use as units the number of cubic millimeters oxygen taken up or carbon dioxide evolved per milligram fresh weight of yeast per hour. If we wished to compare different samples of yeast it would be necessary to use the dry weight, but since in this work the same yeast was used throughout any one experiment the fresh weight may be used. Then any two experiments may be compared on a percentage basis.

Warburg (1926, pp. 8–10) has developed a formula for the calculation of volume changes of gas in the differential manometer. He uses the following quantities:

P = Initial pressure in both vessels.

P_0 = The normal pressure (*i.e.*, 760 mm. Hg) or 10,000 mm. Brodie.

h = Observed difference of level in the manometer fluid.
h' = Increase in pressure in the compensation vessel.
Δp = Increase in pressure in the experimental vessel.
A = Cross section of the capillary.
V_G = Gas space in the experimental vessel.
$V_{G'}$ = Same in compensation vessel.
V_F = Liquid space in the experimental vessel.
$V_{F'}$ = Same in compensation vessel.
T = Absolute temperature.
α = Absorption coefficient of the gas in the experimental vessel.
α' = Same in compensation vessel.
x = cmm. O_2 or CO_2 absorbed or evolved.

The increase of gas in the gas space of the experimental vessel is:

$$\frac{P+\Delta p}{P_o}\frac{273}{T}\left(V_G+A\frac{h}{2}\right)-\frac{P}{P_o}\frac{273}{T}V_G \quad \ldots\ldots(1)$$

If the value of A is small then expression (1) becomes

$$\Delta p\left(\frac{V_G\frac{273}{T}}{P_o}+\frac{A}{2}\frac{273}{T}\frac{P}{P_o}\right) \quad \ldots\ldots(2)$$

He next assumes that P as a rule will equal P_o and therefore $\frac{P}{P_o}=1$. ("Hier ist $\frac{A}{2}\frac{273}{T}\frac{P}{P_o}$, von der Grösse eines Korrektionsgliedes. Bedenken wir, dass P in der Regel nahezu gleich P_o ist, so können wir ohne merklichen Fehler den Faktor $\frac{P}{P_o}$ . . . . gleich 1 setzen" . . . . p. 9.) Therefore expression (2) reduces to:

$$\Delta p\left(\frac{V_G\frac{273}{T}}{P_o}+\frac{A}{2}\frac{273}{T}\right) \quad \ldots\ldots(3)$$

Furthermore the value of Δp is found to be

$$h\left(1+\frac{\frac{A}{2}\cdot\frac{273}{T}}{\frac{V_{G'}\frac{273}{T}V_{F'}\alpha'}{P_o}}\right) \quad \ldots\ldots(4)$$

from which, in the numerator of the fraction within the parenthesis, the term P_o has likewise been eliminated. In dealing with low pressures the assumption obviously does not hold that P approximates P_o. In fact it is usually widely differ-

ent. Hence the factor $\frac{P}{P_o}$ must be retained. The entire equation then is as follows:

$$x = h\left[\left(1 + \frac{\frac{A}{2}\cdot\frac{273}{T}\cdot\frac{P}{P_o}}{\frac{V_G'\frac{273}{T} + V_F'\alpha'}{P_o}}\right)\left(\frac{V_G\frac{273}{T} + V_F}{P_o} + \frac{A}{2}\cdot\frac{273}{T}\cdot\frac{P}{P_o}\right)\right] \quad \ldots\ldots(5)$$

The term enclosed by brackets in expression (5) is the so-called "Gefässkonstant" or "vessel constant" which, when multiplied by the observed change in the manometer fluid level, gives the amount of gas exchange in cubic millimeters. In this term the value P occurs in the numerator twice. Therefore the magnitude of the term will vary directly as P. In other words, as the pressure in the system is lowered the vessel constant will decrease and *vice versa*. Thus if an organism absorbs the same number of molecules of oxygen per unit time as the pressure is lowered then the observed pressure change, h, must increase to compensate for the decrease in the vessel constant.

There are certain technical details which should be noted. The fluid in the manometer was Brodie's solution, used because the value of P_o expressed in millimeters Brodie is an even 10,000. The volumes of the two sides of the manometer were determined by filling each side separately with mercury and weighing. The diameter of the capillary was similarly determined. The stopcocks were greased with a petroleum jelly-rubber composition which was firm enough to prevent leakage of air. Prior to actual use, the whole apparatus was set up empty in the water bath, exhausted as completely as possible, and shaken 2 hours. During this time no change in the fluid level of the manometer could be observed.

RESULTS

The Respiration and Fermentation of Yeast.—When the oxygen uptake of yeast is measured at different tensions the result is similar to that found by other investigators. There is little or no diminution in respiration until quite low tensions are reached. On the other hand, if the *pressure* is reduced the respiration falls off much more rapidly. This is illustrated by the data in Tables I and II.

In Tables I and II it is evident that the oxygen uptake decreases more rapidly when the pressure is reduced than when the pressure is maintained constant and the per cent of oxygen is cut down. A particularly clear instance of this occurs in Table II with a tension of 36 mm. of oxygen. In 5 per cent oxygen at normal pressure the

respiration is 84 per cent of that in air (144 mm.). When the pressure on a mixture containing 21 per cent oxygen, however, is reduced to 190 mm. giving the same partial pressure of oxygen (36 mm.) the respiration is cut down to 34 per cent of the normal. There is then clearly one effect on respiration which is brought about by a reduction of pressure, *per se*, and which is independent of the quantity of oxygen (or tensions).

TABLE I

3 cc. yeast suspension (10 mg. per cubic centimeter). Temperature 37°C. Initial gas mixture 100 per cent oxygen = 712 mm. oxygen tension.[2] 0.4 cc. 10 per cent KOH in inset.

A

Barometric pressure maintained at 760 mm. and per cent of oxygen, exclusive of water vapor, varied by mixing with nitrogen.

Per cent O_2	100	43	21	9	5
Tension O_2 in mm. Hg	712	306	144	64	36
Respiration (cmm. O_2 absorbed per mg. yeast per hour)	48	47.5	47	45	33
Per cent of normal (*i.e.*, of respiration under standard conditions)	100	99	98	94	69

B

Per cent of oxygen maintained at 100 per cent, exclusive of water vapor, and barometric pressure varied.

Barometric pressure in mm. Hg	760	600	500	400	300	200
Corresponding O_2 tension	712	552	452	352	252	152
Respiration (as above)	48	48	44	38	36	16
Per cent of normal (as above)	100	100	92	79	75	33

Since respiration and fermentation (aerobic and anaerobic respiration) are closely related, experiments were performed to find out if the

[2] It is of course necessary in all these experiments to subtract the vapor tension of the water present. At 37°C. this amounts to 48 mm. Hg at all barometric pressures. For example with 100 percent O_2 the total pressure is 760 mm. but the oxygen tension will be 760 − 48 = 712. If this same gas is reduced to a barometric pressure of, say, 300 mm. Hg then the oxygen tension will be 300 − 48 = 252. The same principle applies when the gas mixture contains any other percent of oxygen.

lowering of the pressure affected the latter as well as the former. This might be expected if the reduced pressure attacks the oxidation system of the cell as a whole. If, however, it is confined to that portion of the

TABLE II

3 cc. yeast suspension (10 mg. per cubic centimeter). Temperature 37°C. Initial gas mixture 21 per cent oxygen, 79 per cent nitrogen (exclusive of water vapor). 0.4 cc. 10 per cent KOH in inset.

A

Average of two experiments, expressed as per cent of respiration with the initial mixture as the normal. Barometric pressure maintained at 760 mm. Mixtures as in Table I, *A*.

Per cent O_2	21	9	5	1.6
Tension O_2 in mm. Hg	144	64	36	11.5
Per cent of normal respiration	100	97	84	60

B

Average of five experiments, expressed in per cent of respiration in this mixture at 760 mm. Per cent of gases in the mixture, exclusive of water vapor: oxygen 21 per cent, nitrogen 79 per cent. Barometric pressure varied.

Barometric pressure in mm. Hg	760	380	190	115	80
Corresponding O_2 tension	144	70	30	13	6.5
Per cent normal respiration	100	74	34	27*	7**

* One experiment.

** Average of three experiments.

TABLE III

3 cc. yeast suspension (10 mg. per cubic centimeter). Temperature 37°C. Initial gas nitrogen. Barometric pressure varied.

Barometric pressure in mm. Hg	760	540	415	250	135
Respiration: cmm. CO_2 produced per mg. per hour	45.5	46	45	45	46.5
Per cent of normal (*i.e.*, per cent of respiration under standard conditions	100	101	99	99	102

system which involves simply the uptake of oxygen then there might or might not be an effect on the anaerobic oxidations. Table III is an example of the results obtained. In this type of experiment the

manometer was filled with nitrogen gas which had been run through alkaline pyrogallol to remove all but the last traces of any oxygen that might be present. Such traces as remained would not affect the result materially. No KOH was placed in the vessel and the production of CO_2 was measured.

TABLE IV

3 cc. of 2 per cent fructose (Pfanstiehl) dissolved in N/2 Na_2CO_3. Temperature 37°C.

A

Initial gas 100 per cent O_2. Pressure maintained at 700 mm. Per cent of oxygen varied.

Per cent of oxygen	100	21	9	5	1.6
Oxygen tension in mm. Hg	712	144	64	36	11.5
Cmm. O_2 per hour absorbed by the fructose	530	329	180	81	12.5
Per cent absorption based on 100 per cent O_2 and 760 mm. as 100 per cent	100	62	33	15	2.5

B

Initial gas 100 per cent O_2, exclusive of water vapor, maintained. Pressure varied.

Barometric pressure	760	345	195	80
Oxygen tension in mm. Hg	712	297	147	32
Cmm. O_2 per hour absorbed by fructose	492	369	324	92
Per cent absorption based on 100 per cent O_2 and 760 mm. as 100 per cent	100	73	66	19

C

Both per cent of oxygen in nitrogen, exclusive of water vapor, and the barometric pressure were simultaneously varied so as to bring the oxygen *tension* in all cases to 63 to 64 mm. Hg. The variation in the tension was not over 1 per cent.

Per cent oxygen	9	20	43	100
Barometric pressure in mm. Hg	760	360	190	110
Oxygen tension in mm. Hg	63.5	63	63	63.5
Cmm. oxygen absorbed per hour	213	175	170	188

This experiment, which was checked several times, shows that there is no such diminution of anaerobic respiration with reduced pressure as is experienced with aerobic respiration. In fact there may be said to be no effect at all. The significance of this result is discussed below.

Artificial Systems.—The question arises: Is the different action of

reduced oxygen *tension* and reduced *pressure* due to a purely physical cause having to do with the solution and diffusion of the gas, or is it dependent upon the organization of the cell itself? In this connection the usual type of experiment was performed with two artificial oxidation systems which proceed in homogeneous solution: the absorption of oxygen by fructose in alkaline solutions and the oxidation of cysteine. These systems have been studied exhaustively by Warburg and his co-workers who have shown that both are oxidations catalyzed by metals.[3] (Sakuma, 1923; Warburg and Yabusoe, 1924; Krebs, 1927).

The results are indicated in the data cited in Tables IV and V.

The results shown in Table IV indicate that, apart from experimental error, the amount of oxygen uptake with fructose is dependent on the oxygen tension alone and is not affected materially by

TABLE V

25 mg. cysteine (Pfanstiehl) in 3 cc. borate-KOH buffer at pH 9.3. Temperature 20°C. Initial gas 100 per cent oxygen, exclusive of water vapor.

Per cent oxygen	100	100	43
Barometric pressure in mm. Hg	760	363	760
Oxygen tension in mm. Hg	712	315	306
Cmm. oxygen absorbed per hour per mg. cysteine	25.4	12.6	12.7

changes of pressure. In other words the initial *concentration* of oxygen is the determining factor.

The typical experiment cited in Table V indicates that the cysteine system behaves analogously to the fructose system in that the oxidation is dependent solely on the oxygen tension.

The Inhibition of Respiration by Carbon Monoxide.—The long series of well known facts concerning the action of carbon monoxide on hemoglobin and its capacity to inhibit tissue oxidation (Warburg, 1926) lends support to the idea that we have, in carbon monoxide, a substance which behaves in a manner very similar to oxygen. It

[3] No metals were added to these systems because, although fructose and cysteine of the highest obtainable commercial purity (Pfanstiehl) were used and all possible precautions observed, there were sufficient impurities in the form of metals (chiefly iron) to catalyze the oxidations.

may be said to compete with oxygen in the series of reactions which we call oxidation. It seemed therefore advisable to see if low pressure would prevent the uptake of carbon monoxide as well as that of oxygen. This could, of course, be measured by the *inhibition of the oxygen uptake*. For just so much as the carbon monoxide replaces the oxygen in the oxidation system, by just so much is the normal absorption of oxygen diminished.

The work of Warburg (1926) indicated that when the proportion of carbon monoxide to oxygen is increased the toxicity of the former increases. Since in this investigation it was intended to reduce the pressure on a constant mixture of carbon monoxide and oxygen and thereby decrease the total quantity of both gases, it was of importance to know definitely, apart from the question of pressure, whether the effect of carbon monoxide on oxidation could be varied by keeping the ratio $\frac{CO}{O_2}$ constant and varying the total amounts, or whether, as is implied by Warburg's work, it is solely a matter of the ratio $\frac{CO}{O_2}$. To be certain of the situation two series of experiments were performed: first a series where the ratio of carbon monoxide to oxygen was kept constant but the total quantity of both was altered at 760 mm., and secondly a series where the ratio was altered. In both these series the respiration at a given oxygen tension with carbon monoxide had to be compared with the respiration at the same oxygen tension where nitrogen replaced the carbon monoxide. This, of course, was to control the normal moderate reduction of respiration due to the diminished oxygen tension itself.

In the first series (Table VI) an initial mixture of 21 per cent O_2 and 79 per cent N_2 was used to determine the normal respiration. Then a mixture of 21 per cent O_2, 79 per cent CO was substituted. The latter was then diluted with nitrogen to reduce the total concentration of both oxygen and carbon monoxide to the same degree. In the second series (Table VII) the ratio $\frac{CO}{O_2}$ was changed by filling the manometer successively with different mixtures of the two gases.

Here it will be seen that replacing 79 per cent nitrogen with an equivalent amount of carbon monoxide reduced the oxygen uptake

TABLE VI

3 cc. yeast, 10 mg. per cubic centimeter 0.4 cc. 10 per cent KOH in inset. Temperature 37°C. Initial mixture 21 per cent O_2; 79 per cent N_2, exclusive of water vapor. Pressure maintained at 760 mm. Average of two experiments.

Gas mixture	21% O_2 79% N_2	21% O_2 79% CO	10% O_2 40% CO 50% N_2	5% O_2 20% CO 75% N_2
Oxygen tension in mm. Hg	144	144	72	36
Per cent respiration on basis of 21 per cent O_2 in N_2, as 100 per cent	100	54	50	36

TABLE VII

Comparison of mixtures of oxygen with nitrogen and carbon monoxide. Temperature 37°C. Pressure maintained at 760 mm. Hg. 3 cc. yeast, 10 mg. per cubic centimeter. 0.4 cc. 10 per cent KOH in inset.

Gas mixture	43% O_2 57% N_2	21% O_2 79% N_2	9% O_2 91% N_2	5% O_2 95% N_2
Oxygen tension in mm. Hg	306	144	64	36
Respiration in cmm. oxygen absorbed per hour per mg. yeast	39.7	39.0	37.3	27.5
Per cent respiration on basis of 43 per cent O_2 57 per cent N_2 as 100 per cent	100	98	94	69
Gas mixture	43% O_2 57% CO	21% O_2 79% CO	9% O_2 91% CO	5% O_2 95% CO
Ratio $\frac{CO}{O_2}$	1.33	3.77	10.1	19.0
Oxygen tension in mm. Hg	306	144	64	36
Respiration in cmm. oxygen absorbed per hour per mg. yeast	35.9	27.5	14.8	9.0
Per cent respiration on basis of 43 per cent O_2 57 per cent N_2 as 100 per cent	90.5	69.5	37.4	22.7
Per cent inhibition*	10.5	29.5	60.5	67

* Per cent inhibition is represented by the expression:

$$\frac{100\ (\text{per cent respiration in } N_2 - \text{per cent respiration in CO})}{\text{per cent respiration in } N_2}$$

to about 54 per cent. Further reduction of the concentration of both gases in equal degree at constant pressure causes an added reduction. But this may be accounted for on the basis of reduction of *oxygen*

tension alone. If we call the oxidation in the presence of 21 per cent O_2, 79 per cent CO equal to 100 per cent then with 10 per cent O_2, 40 per cent CO it is 92.5 per cent and with 5 per cent O_2, 20 per cent CO it is 67 per cent. This is slightly greater than, but of the same order of magnitude as, what would be expected on the basis of the oxygen-nitrogen mixtures previously investigated (Table I). Therefore it may be said that if the ratio remains constant the inhibition remains sensibly the same.

What happens when the ratio is altered may be seen in Table VII where it is very evident that the inhibitory effect of carbon monoxide

TABLE VIII

3 cc. yeast (10 mg. per cubic centimeter). Initial mixtures 21 per cent O_2, 79 per cent N_2 and 21 per cent O_2, 79 per cent CO (exclusive water vapor). Pressure varied. Temperature 37°C. Average of four experiments.

Gas mixture	21% O_2 79% N_2	same	same	same
Barometric pressure in mm. Hg	760	380	190	80
Oxygen tension in mm. Hg	144	70	30	6.5
Per cent respiration on basis of 21 per cent O_2, 79 per cent N_2 as 100 per cent.	100	71	31	7.5
Gas mixture	21% O_2 79% CO	same	same	same
Barometric pressure in mm. Hg	760	380	190	80
Oxygen tension in mm. Hg	144	70	30	6.5
Per cent respiration on basis of 21 per cent O_2, 79 per cent N_2 as 100 per cent.	56	46	27.5	8
Per cent inhibition	44	36	14	0

increases as the ratio $\frac{CO}{O_2}$ increases and *vice versa*. When the pressure is reduced on a constant mixture of two such gases of low solubility their ratio in solution will remain practically constant. If the reduced pressure has an equal effect on the reactions of the cell itself with respect to the two gases the degree of inhibition on the part of the carbon monoxide will not be altered. If on the other hand, the degree of inhibition is altered it may be taken as an indication that low pressures affect the reactions of the two gases differentially in the cell.

We may now proceed to what happens when the pressure is reduced.

The data covering this situation are summarized in Table VIII. In all experiments two parallel series were run, first with 21 per cent O_2, 79 per cent N_2, then, on the same yeast with 21 per cent O_2, 79 per cent CO. The inhibition due to the carbon monoxide was then calculated.

From Table VIII one significant conclusion may be drawn. When the pressure is reduced the inhibition progressively disappears until it may be said to be entirely removed. In others words, low pressures have a greater effect on carbon monoxide than on oxygen with respect to their relative power to affect the oxidation system of the cell.

DISCUSSION

There are certain theoretical considerations which may be discussed with regard to the foregoing experimental evidence without attempting to build up any complete hypothesis to account for the effect of low pressures on cell oxidations.

1. That living oxidation systems are affected by low pressures to the exclusion of artificial systems has been shown by the fact that the respiration of yeast is reduced by low pressures over and above what can be accounted for by simple decrease in oxygen concentration (tension) whereas such low pressures seem to have no *extra* effect whatever on artificial systems of the fructose or cysteine type. The rate of the latter is governed solely by the number of oxygen molecules present. This proves nothing but what is already universally admitted, that in the complex and heterogeneous oxidation system of the cell there is a mechanism which cannot be duplicated in all respects by a relatively simple, homogeneous, system *in vitro*. We may however, narrow down the field to some extent, for any purely concentration effects due to the action of reduced pressure on a homogeneous system may be ruled out.

2. The lack of any influence of low pressures on the anaerobic respiration of the yeast indicates that not all of the oxidation system of the cell is affected, but only that part which has to do with the uptake of oxygen. We must assume the existence of some kind of catalyst in the cell which facilitates this uptake either by direct union with oxygen, or by causing it to combine, temporarily at least, with some substance from which the oxygen can be carried to the final

oxidisable materials in the cell. The possibility of an organic peroxide immediately suggests itself, or a haem derivative on the order of hemoglobin or Warburg's "Atmungsferment." It is not necessary to postulate any definite chemical entity but is sufficient to assume merely the presence of some substance with which the oxygen combines.

It is known that some peroxides such as hydrogen peroxide are relatively stable in contact with inert gases like nitrogen at normal pressure, but are very easily decomposed if the pressure is lowered. The equilibrium between hemoglobin and the oxygen tension has likewise been elucidated although in this instance it is generally understood that the equilibrium is the same regardless how the partial pressure of the oxygen is achieved (whether by gas mixtures or by reduction of the barometric pressure). At all events the suggestion may be made that one of the intermediate compounds of oxygen in the cell is of such a nature that it is broken up to a greater or less extent by lowering the barometric pressure. Conversely, of course, a low pressure would prevent the formation of some of this compound in the first place and would thus decrease the amount of oxygen which could eventually be used by the cell.

3. The data secured with carbon monoxide are of interest in this connection. Since it has been shown quite definitely that the degree of inhibition on the part of carbon monoxide depends upon the ratio $\frac{CO}{O_2}$ the differential effect of low pressure must mean that, in the cell itself, at the point where oxidation is taking place, the ratio must be altered by low pressure. If we make the further assumption that the intermediate compound previously mentioned may be formed with carbon monoxide as well as oxygen then we have only to say that low pressures decompose the compound with carbon monoxide more readily than that with oxygen. Warburg and Negelein (1928) have proved quite conclusively that there exists a CO- "Atmungsferment" compound which is decomposed by light. This is itself evidence of a relatively unstable compound. It seems also reasonable to suppose that this, or a similar, substance may be decomposed by low pressure.

SUMMARY

1. A method is described for measuring tissue oxidation under reduced barometric pressure.

2. The oxygen uptake of yeast is diminished by low barometric pressures to a greater extent than by a reduction of the partial pressure of oxygen, to a corresponding degree, at atmospheric pressure.

3. This effect of low pressure is not observed with certain *in vitro* oxidation systems.

4. The anaerobic respiration (carbon dioxide production) of yeast is not at all affected by low pressures.

5. The inhibition of tissue oxidation caused by carbon monoxide is removed by lowering the pressure.

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CHEMICAL STIMULATION BY ALCOHOLS IN THE BARNACLE, THE FROG AND PLANARIA

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The several types of stimulation[1] are usually classified according to the nature of the environmental agent acting upon a receptor, and the classificatory terms "photoreception," "mechanical excitation," or "chemical stimulation" are used merely to indicate that the stimulation has been initiated by a certain type of stimulating agent. Subsequent events within the receptor may or may not be similar to the first effect at the receptive surface. All of the processes at the receptive surface and within the receptor involve the redistribution or the transformation of energy. It should therefore be possible to analyse the successive steps in stimulation and reception by tracing the energy changes. Studies on photic stimulation (Hecht, 1919–20, 1920–21, 1922–23, 1926–27, 1927–28, 1928–29, and 1929, and papers quoted therein), and on mechanical stimulation (Crozier, 1923–24; Crozier and Pincus, 1926–27, 1927–28, 1929–30; Crozier and Stier, 1927–28, 1928–29 and papers quoted therein; Adrian and Zotterman, 1926, and

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[1] "Stimulation" as used here refers to the first effects of an environmental agent upon a receptive surface, which initiate a catenary series of processes within the receptor. The final result of such receptor processes is the release of energy in some form to an adjacent material, membrane, or other cellular or non-cellular structure, which in turn transmits effects bringing about the eventual "response" or "reaction." In animals with a nervous system, the receptor processes eventually release nervous impulses.

papers quoted therein), have furnished generalized principles upon which interpretative analyses of these types of stimulation may be based. For chemical stimulation it has been difficult to formulate such principles satisfactorily, because of the incompleteness of suitable data, and because of the confusing and contradictory nature of the data already reported.

In our attempt to secure data on chemical stimulation we have studied the effects of the simplest alcohols (the normal primary aliphatic alcohols) on the barnacle, the frog, and *Planaria*, under experimental conditions which do not involve secondary processes, such as simultaneous stimulation by other agents, adaptation, narcosis, and the like. If the results are interpretable and lead to a logical hypothesis, it should be possible to test the hypothesis by investigating other homologous series of organic compounds. Such a procedure may lead to a generalized statement concerning the nature of the processes involved in chemical stimulation.

Methods

For determining the stimulating efficiencies of the first four normal primary aliphatic alcohols on *Balanus tintinabulum*,[2] groups of young specimens from 2 to 10 mm. in basal diameter, and from 6 to 9 mm. in height, seated upon a stone or shell, were placed in a dish of running sea water as described in a previous paper on the effect of temperature in *B. balanoides* (Cole, 1928–29). The animals were properly protected from all other kinds of environmental stimuli. The sea water and the alcoholic solutions (made with sea water in 10 liter amounts) were run through a tin coil immersed in a water bath the temperature of which was held at 18.0°C. ±0.5°. After observing that the pedal rhythm was normal in character and rate, the flow of sea water was stopped and the experimental solution was turned on at the same rate of flow (250 cc. per minute). The criterion for stimulating effect was any change in the rhythmic movements of the cirri. The nature of the change was noted and recorded, as well as the time within which it occurred. Sea water then replaced the solution and was allowed to flow at least 20 minutes before another test was made. (Full recovery from effects of the alcohols used was apparent by the end of 10 minutes.) By this method the threshold stimulating concentration of each alcohol was determined. It may be assumed that the alcohol furnished the minimum amount of energy necessary to "activate" or stimulate the

[2] The experiments on *Balanus* were done at the Hopkins Marine Station, Pacific Grove, Calif., to the Director of which the senior author is indebted for many courtesies.

receptor. In all cases the time within which any irregularity in the cirral rhythm appeared was practically constant (from 10 to 20 minutes). The order of alcohols used was as follows: ethanol, propanol, methanol, butanol. The tests were repeated several times over a period of 3 weeks on three different groups of animals (250 individuals in all).

Studies on the stimulating efficiencies of the first five normal primary aliphatic alcohols were made in an analogous manner on the frog *Rana pipiens*. The frog was placed in a glass dish shielded from mechanical and photic stimuli. Tap water or the experimental solution flowed through the dish at a rate of 250 cc. ±15 cc. per minute, and at a temperature of 18.0°C. ±0.3°. The outflow was so adjusted that the liquid in the dish was 5 mm. deep. After 1 hour's acclimatization to the experimental conditions, during which "spontaneous" movements[3] were rare, the flow of tap water was stopped and the alcoholic solution (made up in distilled water in 3 liter amounts) started. As soon as a movement of the frog occurred the solution was replaced by tap water and the frog's skin was thoroughly washed by allowing excess water to flow through the dish. The reaction time was measured by a stop watch to 0.1 second. An interval of at least 30 minutes with tap water flowing at 250 cc. per minute was then allowed before the next test was made. Periods of much less than 30 minutes were too short to prevent adaptation of the frog to the alcohol. For example, if only 15 minutes were allowed between trials, the reaction time of the frog steadily increased until finally no response at all occurred. Since all areas of the skin exposed to the solutions were not equally sensitive, and since distribution of the solution throughout the dish was altered by changes in position of the frog, it was necessary to limit the orientation of the frog's body in respect to the current. The postero-anterior axis of the body coincided with the direction of flow. Slight change from this position resulted in increased variation in the reaction times obtained. Preliminary trials showed that all disturbing effects of simultaneous stimuli and of adaptation were reduced to a minimum by the procedure described. The response of the frog consisted of a vigorous movement of the hind legs and could rarely be confused with the so-called spontaneous movements. Among different frogs different thresholds of stimulation were usually found, but since the ratios of reaction times from any two frogs were constant it was possible to use the data obtained from different individuals by merely reducing the reactions times to a common threshold. After preliminary trials to determine a concentration of ethanol which was near the threshold value and which gave a reaction time of about 20 seconds, a series of solutions of the five alcohols[4] was made up beginning with methanol at 1.8 molar

[3] "Spontaneous" movements are defined as any movements due to uncontrollable external or internal stimuli.

[4] Only alcohols of the highest purity from Eastman Kodak Company were used. Specific gravities were determined and were found to coincide with the best values reported in the literature.

and decreasing in molarity by one-third for each successive alcohol. Tests were then made with the five alcohols in different orders, using several different frogs over a period of 6 months. A series was run with decerebrate frogs to detect any difference in behavior. No significant variation in the results was noticeable, so that normal frogs were later used exclusively. A final series of tests were made on the frog with methanol to discover the effects of varying the concentration on the reaction time.[5]

Using the same method as for the frog, the effects of the alcohols on *Planaria dorotocephala* were also studied.[6] A smaller experimental dish was used, through which the water and the alcoholic solutions flowed at the rate of 20 ±5 cc. per minute. The temperature was 18.0 ±0.3°C. After demonstrating that *Planaria* exhibited a definite response whenever an alcoholic solution of a high enough concentration passed over it, tests were made with each of the five alcohols, at five different concentrations. The criterion of response was the beginning of locomotion. If the *Planaria* were allowed several hours of acclimatization, they remained quiescent thereafter unless stimulated. Soon after the alcohol came in contact with them, locomotion began, the reaction time depending upon the concentration of the alcohol. A complete series of tests involving at least 4 observations at each of five concentrations of each alcohol on single animals yielded uniform results. Marked variations among different individuals made it difficult to interpret the results obtained from a population of *Planaria*.

DISCUSSION OF RESULTS

The data from the barnacle presented in Table I and plotted in Fig. 1 indicate that the stimulating efficiencies of the successive alcohols increase about 3-fold as each CH_2 group is added to the molecule, beginning with methanol. This result is analogous to the findings of Traube and others, who have studied the narcotic efficiency of alcohols and other organic compounds. (For review of the literature consult: Traube, 1927.) According to Traube's theory the concentrations of members of a homologous series of organic compounds, such as the normal primary aliphatic alcohols, necessary to produce a given effect will vary as the following geometrical series, $1: 3^{-1}: 3^{-2}: 3^{-3}: 3^{-4}. \ldots$ Such an exponential relationship may be expressed by the following equation—

$$C_1 = C_2 a^n \qquad (1)$$

[5] For these experiments we are indebted to Mr. Leslie A. Stauber.

[6] All of the tests on *Planaria* were made by Mr. Alfred Margolis, to whom grateful acknowledgment is made.

where C_1 is the concentration of a selected member of the series necessary to give a definite reaction, C_2 is the concentration of a higher member of the series necessary to produce the same effect, a is a constant

TABLE I

Minimum stimulating concentrations of alcohols for *Balanus*. Temp. = 18.0 ±0.5°C.

Alcohol	Threshold concentration	Concentration calculated from equation (1) in text
Methanol	0.06M	(0.06)M
Ethanol	0.017	0.02
Propanol	0.0067	0.0066
Butanol	0.0027	0.0022

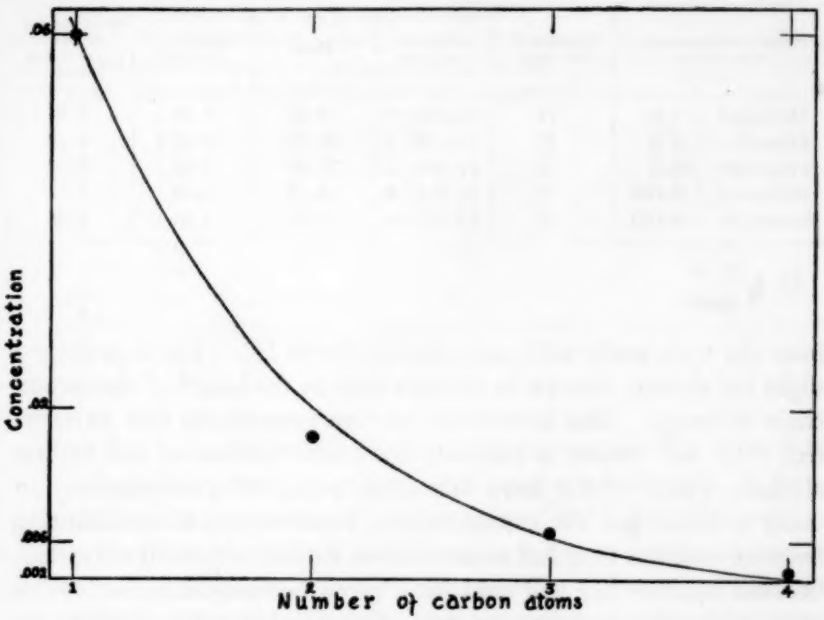

FIG. 1. Threshold stimulating concentrations of methanol, ethanol, propanol and butanol for the barnacle plotted againt the number of carbon atoms in the respective molecules. The curve is exponential with the base equal to 3. The circles represent the experimental concentrations, showing very close agreement with the theoretical values.

expressing the ratio of successive concentrations, and n is the difference in the number of carbon atoms in the two molecules. The minimum stimulating concentrations of the alcohols for the barnacle calculated from equation (1), when a is 3, are very close to the threshold concentrations determined experimentally.

Proceeding on the assumption that a similar ratio would also hold for the stimulating efficiencies of the alcohols on the frog, concentrations were calculated from equation (1) and the effects of the solutions were tested.[7] That the receptive process measured was reversible was indicated by the relative constancy of the reaction times obtained

TABLE II

Reaction times of frog to alcohols at concentrations decreasing by one-third. Temp. = 18.0 ±0.3°C.

Alcohol and concentration—molar		Number of reactions	Extreme variations	Mean	Mean error of mean*	Mean error of mean as percentage of mean
Methanol	1.8	25	11.2–30.0	18.82	1.04	5.54
Ethanol	0.6	25	13.2–28.3	20.90	0.98	4.70
Propanol	0.2	25	11.9–33.2	21.30	1.37	6.44
Butanol	0.066	25	11.5–32.0	24.51	0.94	3.87
Pentanol	0.022	25	15.5–33.4	24.86	1.11	4.46

*$\pm\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

from the tests made with each alcohol (Table II). The data show a slight but definite increase in reaction time as the length of the carbon chain increases. This increase in reaction time means that an incorrect ratio was chosen to calculate the concentrations of the various alcohols which would have the same stimulating efficiencies. In order to determine the correct ratio a knowledge of the relationship between reaction time and concentration for each alcohol is necessary, because equation (1) will hold for a given homologous series over a wide range of concentrations only if that relationship is relatively constant throughout the series. In Table III is presented the data

[7] A preliminary report of incomplete experiments appeared recently (Cole and Allison, 1929–30).

obtained on the frog by varying the concentration of methanol. The graphical representation of this data in Fig. 2 indicates a linear relationship between log concentration and the reciprocal of the reaction time over the range of concentrations studied, according to the following equation:

$$B = t \log \frac{C}{A} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where C is the concentration necessary to produce a definite reaction in time t, A is the antilogarithm of the Y-intercept, and B is the slope

TABLE III

Reaction times of frog to different concentrations of methanol. Temp. = 18.0 ±0.3°C.

Number of reactions in each case was 15.

Concentration—molarity	Mean reaction time—seconds	Mean error of mean as percentage of mean*
1.6	38.9	13.31
1.8	29.8	9.76
2.0	23.3	11.85
2.2	18.8	6.49
2.4	20.3	9.06
2.6	17.4	13.55
2.8	11.9	6.12
3.0	11.4	5.15

* $\frac{100}{\text{Mean}} \times \sqrt{\frac{\Sigma\, d^2}{n(n-1)}}$

of the line. A series of similar concentration studies with each of the five alcohols on *Planaria* exhibited the same relationship except at the highest concentration of each alcohol (Table IV). In order to make the plots more nearly coincident, as in Fig. 3 the concentrations were multiplied by the ordinate factor given in Column 5 of Table IV. At the highest concentration, the rates of reaction are slower than would be predicted from the relationship revealed by the four lower concentrations. That these deviations are real and significant is shown by the small percentage errors. Although an interpretation of this effect

is impossible without further study, it may be suggested that secondary processes at the receptor surface are responsible for the slower rates.

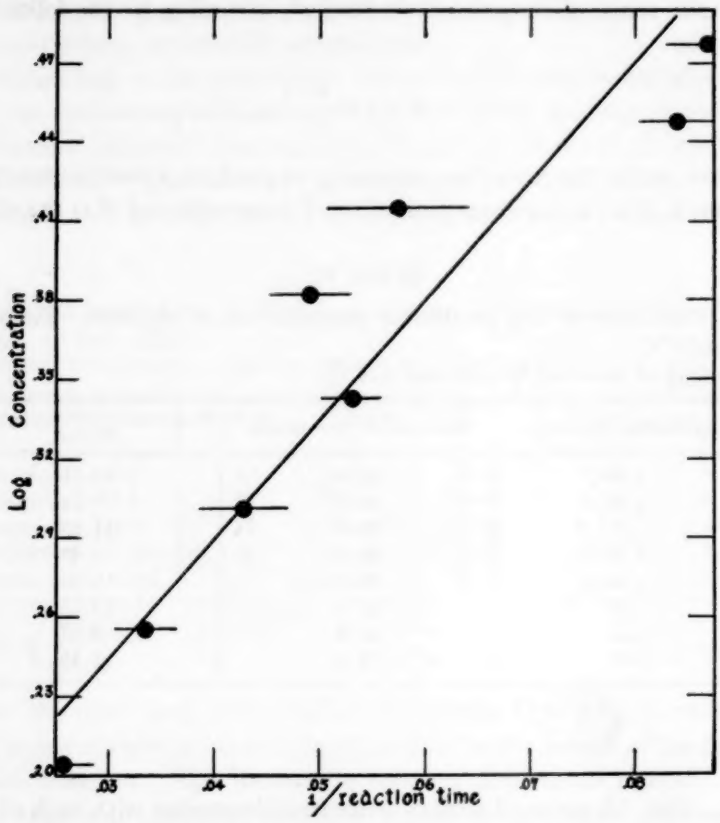

FIG. 2. Plot of reciprocal of reaction time of frog to methanol against logarithm of concentration. The length of the horizontal lines through the circles represents the magnitude of the mean error of the mean expressed as percentage of the mean. The equation of the line is $B = t \log \frac{C}{A}$, where the slope, B, = 4.5 and A, the anti-log of the Y-intercept, = 1.285.

If equation (2) is assumed valid for all the alcohols then the effect produced and measured is constant for all the alcohols, and is propor-

tional to B. The value of B is therefore characteristic of the receptive process initiated by members of the alcohol series in any one animal,

TABLE IV

Reaction times of *Planaria* to different concentrations of five normal primary aliphatic alcohols. Temp. = 18.0 ±0.3°C.

Alcohol and concentration —molarity		Number of reactions	Mean reaction time—seconds	Mean error of mean as percentage of mean*	Ordinate factor
Methanol	0.55	6	26.3	7.31	
	0.60	11	19.1	6.33	
	0.65	13	17.0	6.77	1.000
	0.75	8	12.2	5.16	
	0.85	9	11.5	2.96	
Ethanol	0.183	6	28.8	9.20	
	0.200	9	19.7	3.91	
	0.216	5	16.0	6.63	2.949
	0.250	6	11.9	5.63	
	0.283	6	11.7	3.16	
Propanol	0.0611	4	25.5	9.06	
	0.0666	6	19.2	5.94	
	0.0722	4	13.7	7.01	$(3.045)^3$
	0.0833	6	11.5	2.78	
	0.0944	5	11.1	1.98	
Butanol	0.0203	5	29.3	10.04	
	0.0222	9	18.8	4.95	
	0.0240	4	17.2	5.64	$(2.999)^3$
	0.0277	6	12.1	3.63	
	0.0315	7	9.8	2.66	
Pentanol	0.0068	5	25.0	4.26	
	0.0074	8	18.4	6.58	
	0.0080	5	14.0	4.00	$(3.038)^4$
	0.0092	6	10.6	2.16	
	0.0105	10	10.5	2.19	

$$\frac{100}{\text{Mean}} \times \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

while A is a constant for each alcohol, decreasing in value as the number of carbon atoms increases. From equations (1) and (2), it is apparent that

$$a = \frac{A_1}{A_2} = \frac{A_2}{A_3} = \frac{A_3}{A_4} = \frac{A_4}{A_5}$$

From the plots in Figs. 2 and 3 the values of B for the frog and *Planaria* were found to be 4.50 and 2.80 respectively. By substituting

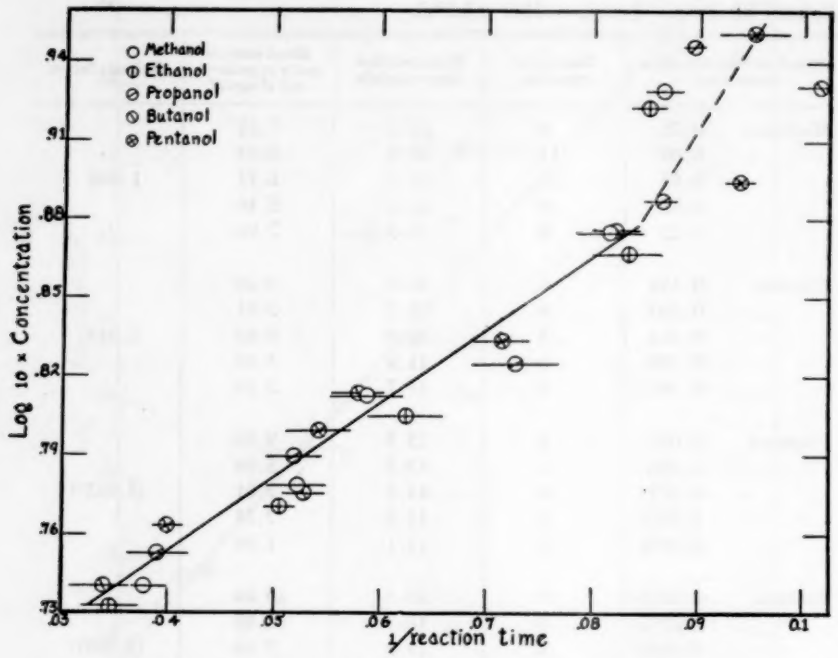

FIG. 3. Plot of reciprocal of reaction time of *Planaria* to five alcohols against the logarithm of 10 × concentration. The length of the horizontal lines through the circles represents the magnitude of the mean error of the mean expressed as percentage of the mean. The concentrations have been multiplied by the respective ordinate factors given in Column 5 of Table IV. The equation of the line is $B = t \log \frac{C}{A}$ where the slope, B, = 2.8 and A, the antilog of the Y-intercept, = 0.4376.

them in equation (2), A for each alcohol and each animal was then calculated from the data, and the ratios between successive A's were determined. These values, as presented in Table V, indicate that the ratio 3 is correct for *Planaria*, but slightly too high for the frog. Such

a difference between animals may be expected since there is no *a priori* reason why the ratio for stimulating efficiency of successive members of a homologous series should be the same for all animals.

It has been assumed that the "rate of the reaction" measured in our studies is proportional to the amount of energy change at the receptive surface, and that this change is determined by the non-polar portion of the molecule, or by the length of the carbon chain. A generalized theory to account for energy changes at interfaces on the basis of a comparison between the activities of the more active and the less active portions of polar organic molecules has been developed by Langmuir (1917, 1925, 1926, 1929) and by Harkins (1924, 1926).

TABLE V

Values of A in equation (2) for the frog and for *Planaria*, when $B = 4.5$ and 2.8 respectively.

Alcohol	Frog		*Planaria*	
	A	$\frac{A_1}{A_2}$	A	$\frac{A_1}{A_2}$
Methanol	1.0380		0.4359	
Ethanol	0.3655	2.84	0.1449	3.008
Propanol	0.1229	2.97	0.0468	3.091
Butanol	0.0432	2.84	0.0161	2.898
Pentanol	0.0145	2.98	0.0051	3.157
		Ave. 2.90		Ave. 3.038

According to Langmuir's "principle of independent surface action," it is concluded that the "forces acting between molecules of various types" among a large class of substances are "dependent mainly on the nature of the surfaces of the molecules which are in contact." For example, in an air-water interface organic molecules orient in such a way that the polar groups are directed towards the water and the less active groups away from the water. At a liquid-liquid interface molecules of an organic solute will be oriented according to the attraction or repulsion of each liquid for certain groups in the molecules of the solute. At an interface between an organic liquid and water the polar groups of the solute molecules will be directed towards the water and the non-polar parts will be drawn towards the organic

liquid. At a receptive surface in animals, therefore, the particular orientation of a polar organic molecule which is stimulatory will determine the nature of the stimulation process. In a homologous series of polar oganic compounds the efficiency for stimulating the receptor will be determined either by the potential of the polar groups, or by the structure and magnitude of the non-polar groups, whenever one or the other becomes dominant in its effect. A similar interpretation has been made by Crozier (1918) in an illuminating discussion of the factors involved in stimulation by fatty acids.

Hixon and Johns (1927) have arranged organic radicals in an activity series based upon the variations of the polar properties of compounds in any series, such as R-OH, R-COOH, R-NH_2, etc. A similar series could also be arranged for the stimulating efficiency of its members if the effect produced were solely a function of the potential of the polar group. For example, if the stimulating efficiency depends upon the hydrogen ion concentration resulting from the dissociation of the carboxyl group, then the radical attached to that group would exert its effect only through the changes it might produce on the dissociation constant. Crozier (1916) has clearly shown that in certain acid series, where the dissociation constants are high, the penetration efficiency of members of the series through a living membrane is chiefly a function of the hydrogen ion concentration. However, it is to be expected that the anion and any undissociated molecules would also exert some effect especially in stimulation where changes in surface energy may be a dominant factor. From a similar study made on the stimulating efficiency of acids, Crozier (1916) states: "The strong acids follow the order of their ionization, but this at such high dilutions that the correspondence probably has little real reference to dissociation." Warburg (1921) has studied the effect of N/100 solutions of acetic aldehyde, proprionic aldehyde, butyric aldehyde and valeric aldehyde on the respiration of blood cells. He found that equal concentrations of these compounds were necessary to reduce respiration by 50 per cent. He concludes that the hindrance to respiration is caused by the specific aldehyde group. If the potential of the polar group of the aliphatic aldehydes used in Warburg's experiments is relatively constant, and if the effect produced by these compounds is primarily due to a reaction of the aldehyde group with some vital constituent in the

cells, equivalent concentrations should produce similar results. Although Warburg's work on respiration is not strictly analogous to the results presented in this paper, it agrees with the interpretation of studies on stimulation.

If the potential of the polar group in any series is relatively constant and primarily effective through its influence upon the orientation of a member of a series at the receptor surface, then the stimulating efficiency of that member may be related to the structure of the less active portion of the molecule. Langmuir (1917) has calculated the loss in potential energy, λ, when a gram molecule of an organic substance passes from the interior to the surface film formed between air and water, from the equation

$$\lambda_{cal} = \lambda_o + 625\, n$$

where λ_o has a different value for each type of compound, and n equals the number of carbon atoms. In other words, in any homologous series the effect of the addition of each CH_2 group upon λ_o is constant. Thus in the normal aliphatic alcohols, ester or fatty acids series, where the potential of the polar group is almost constant, the degree of stimulation should increase as the length of the carbon chain increases, provided the more active portion of the molecule functions primarily as an orienting group. Crozier (1916) has shown that when a low hydrogen ion concentration is approached, as in the monobasic fatty acid series, the dominant factor in determining the degree of stimulation is the length of the carbon chain. To this rule formic acid was an exception. In his discussion of the acid penetration series, Crozier pointed out that formic acid has a higher dissociation constant than its homologues. While discussing the effect of additional CH_2 groups upon the field of force around a carboxyl group in the fatty acid series, Langmuir (1929) states: "The forces near the surface of the carboxyl group should be practically constant for all acids higher than proprionic acid, and would be roughly the same in the case of acetic acid, but might be considerably different in the case of formic acid." According to Hixon and Johns (1927), this variation might also be expressed in terms of λ, since "if the orientation of the molecules is a function of the polar group, $\Delta\lambda$ for any homologous series would not be constant

unless the potential of this group remained unchanged as CH_2 units were interposed." If a relatively slight change in the potential of the polar group may produce an added stimulating effect it might be expected that only where $\Delta\lambda$ was actually a constant would the order of stimulating efficiency be determined by the number of intervening CH_2 units in the carbon chain of any homologous series. On the other hand, if such a change in the potential of a polar group in any homologous series is primarily effective for stimulation through its influence upon orientation of the molecules in a surface, the first member of the fatty acid series would probably not be much of an exception to the rule. This statement is based upon Langmuir's interpretation of the effect of the fatty acids upon the surface tension of water, where formic acid is not considered an exception. For example, Loeb (1908–09) found the following order of efficiency for the normal fatty acids in membrane formation in the sea urchin egg:

butyric > proprionic > acetic > formic

Formic acid is here less effective than acetic, so it may be assumed that membrane formation by fatty acids, including formic acid, is primarily due to a change in surface energy induced by the orientation of the molecules of the members of that series at the surface of the egg.

In the aliphatic alcohol series, the potential of the polar group in the different molecules remains practically constant throughout, and is primarily effective for stimulation through its influence upon surface orientation. It would be expected therefore that the degree of stimulation by successive alcohols would reveal a definite relationship to the structure and magnitude of the non-polar groups in the molecules. This relationship is exponential for the barnacle, the frog, and *Planaria*, and may be mathematically expressed. The formula derived contains two constants, one of which is characteristic of the process of stimulation by alcohol in any one animal. The other constant characterizes each alcohol, the ratio of the values from successive alcohols being identical with the ratio of successive concentrations necessary to produce a given effect. Although the exact nature of stimulation by alcohols is not yet revealed, there is provided an experimental and theoretical foundation upon which further studies of homologous

series may be based. It is hoped that progress can thus be made towards an analysis of the initial energy changes involved in chemical stimulation.

SUMMARY

1. The stimulating efficiencies of some normal primary aliphatic alcohols have been determined for the barnacle, the frog, and *Planaria*, under conditions which do not involve narcosis or simultaneous stimulation by other agents.

2. Concentrations of the successive alcohols necessary to produce a given stimulatory effect vary according to the following geometrical series: 1: a^{-1}: a^{-2}: a^{-3}: a^{-4}: . . . ., where a represents some real number.

3. Within certain limits the relationship between the logarithm of the concentration necessary to produce a given effect and the reciprocal of the reaction time is linear in the frog and in *Planaria*.

4. The concentration effect may be expressed by an equation which contains one constant characteristic of the alcohol series, and another one characteristic of each member. The ratio of the latter constants for successive alcohols represents a in the above series.

5. The stimulation by alcohols in these animals is considered to be due to energy changes at the receptive surfaces, brought about by a definite orientation of the respective alcohol molecules. Increase in stimulating efficiency as the number of CH_2 groups increase must be due to the rôle of the non-polar portion of the alcohol molecule, since the polar group remains practically constant throughout the series.

6. In homologous series of organic compounds it is conceived that stimulating effects will be produced either by the polar group or the non-polar group, according to which one becomes dominant in effect, or to a combination of the two.

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CORRECTION

In Vol. **14**, No. 1, September 20, 1930, page 85, in the next to the last line, under References, for *Amer. J. Physiol., 1918, 14, 315*; read *Amer. J. Physiol., 1918, 45, 323.*

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THE ARGININE AND PREARGININE GROUPS IN EDESTIN

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I

INTRODUCTION

Evidence was given in 1928[1] to show that the combination of strong acid or alkali with a protein is produced by forming salts with the "extra groups" of those trivalent amino acids which can be isolated from the protein (namely, aspartic acid, glutamic acid, tyrosine, histidine, lysine, and arginine). The "titration curve" of a protein corresponds approximately to the amounts of these various groups, each with its typical titration index.[2]

Arginine, however, formed a marked exception. It appeared to contribute to the titration curve in much smaller amount than is found on hydrolysis. On the other hand, a group of unknown origin having a titration index of pH 4.6 was present in amounts equal to the deficiency in arginine.

The name *prearginine* was given to this group on the assumption that it is a chemical group which ionizes at pH 4.6 in proteins but yields *arginine* on hydrolysis of the protein.

It has subsequently developed that the index 8.1 assumed for the "extra group" of *arginine* was incorrect (being based on data obtained

[1] Simms, H. S., *J. Gen. Physiol.*, 1928, **11**, 629; 1928, **12**, 231.

[2] A "dissociation index" is: $pK' = -\log K'$ where K' is the apparent dissociation constant of a weak electrolyte (referred to the hydrogen ion). The dissociation index represents the pH value at which the group is half ionized.

The "titration indices" (pG') determine the titration curve and cannot be correctly called "dissociation indices" although there is little numerical difference between the titration constants (G') and the dissociation constants (K'). The use of these two sets of constants and indices is confusing but unavoidable. See Simms, H. S., *J. Am. Chem. Soc.*, 1926, **48**, 1239.

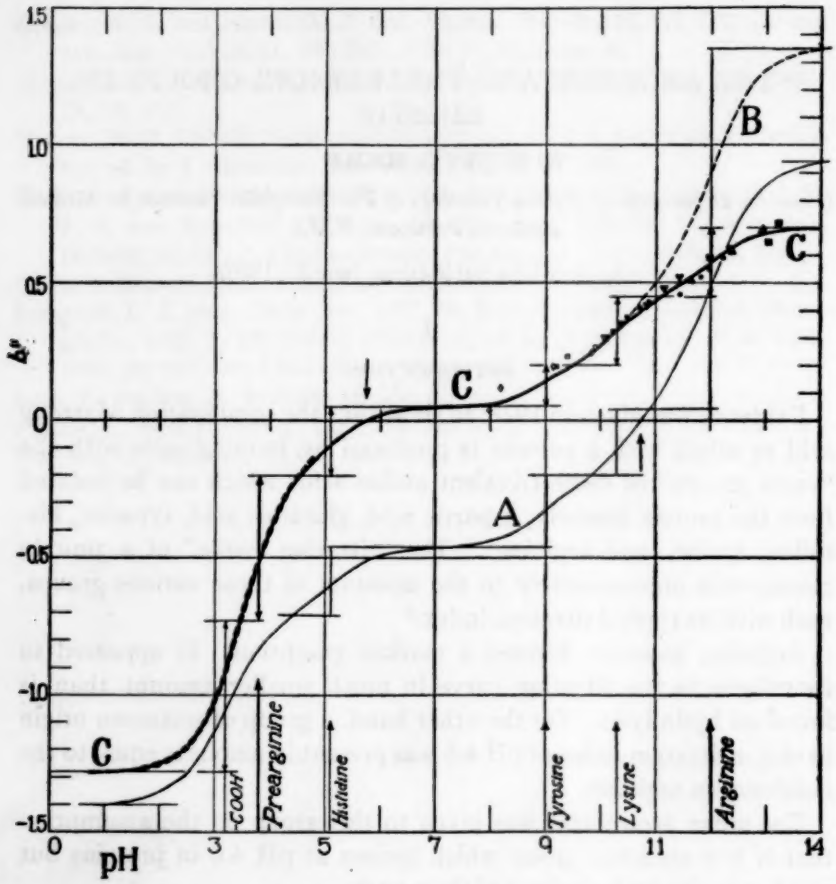

FIG. 1. Titration curve of edestin. The solid symbols represent data from clear solutions; the open symbols, from cloudy solutions. The heavy dash line is drawn to fit the data in acid solution of Hitchcock and of Kodama.

Curve *A* is calculated from the amino acids found on hydrolysis.

Curve *B* (which branches off from *C*) represents the way edestin would ionize if it had the above groups (found on hydrolysis), but in addition had an *extra* group at pH 3.8.

Curve *C* corresponds with the data in Column 5 of Table I.

from impure material). Schmidt[3] has obtained the value $pK'_3 = 12.5$ which agrees with that of Hunter and Borsook ($pK'_3 = 13.85$ at 18°C.).

[3] Schmidt, L. A., Kirk, P. L., and Appleman, W. K., *J. Biol. Chem.*, 1930, **88**, 285.

We have repeated our titration of arginine and corroborate the value of Schmidt.

In order to determine whether this would alter the above theory it was necessary to obtain more accurate data than were available, on a protein in very alkaline solution. Edestin was again chosen due to its high content in arginine.

II

RESULTS

A number of experiments were performed with edestin in solutions up to 14 per cent and at pH values up to 13.5. A method was adopted which as far as possible caused the unavoidable errors to cancel out. The data are given in Fig. 1 (and Table II).

An analysis of these data is given in Table I.

TABLE I

(1)	(2)	(3)	(4)	(5)	(6)
Type	Groups	Indices in edestin	Equivalents per 1000 gm.		
			On hydrolysis	Titration data	Difference
Acidic	Dicarboxylic acids	$pG_1' = 3.2$	0.72	0.55	−0.14
	Tyrosine	$pG_4' = 9.0$	.25	.15	−.10
	Sum		0.97	0.70	
Basic	PREARGININE	$pG_2' = 3.8$	0	0.53	+0.53
	Histidine	$pG_3' = 5.1$	0.25	.25	0
	Lysine	$pG_5' = 10.3$	.25	.25	0
	ARGININE	$pG_6' = 12.0$	.90	.25	−0.65
	Sum		1.40	1.28	
Sum of prearginine plus arginine			0.90	0.78	

Values in Column 4 are those listed by Mitchell and Hamilton,[4] except for histidine and lysine in which case the values of Van Slyke[5] are taken. The figure for dicarboxylic acids represents aspartic acid (0.76) plus glutamic acid (1.31) minus ammonia (1.35) and represents the carboxyl groups not bound by ammonia.

[4] Mitchell and Hamilton, The Biochemistry of the Amino Acids, Chem. Cat. Co., 1929, 191.

[5] Van Slyke, D. D., *J. Biol. Chem.*, 1911, **10**, 15.

If edestin contained the free groups found on hydrolysis (Table I, Column 4) it would have a titration curve represented by Curve *A* in Fig. 1. This is obviously not the case.

If edestin had approximately the above free groups, and *in addition* a group with an index at pH 3.8, in proper amount, the titration curve would be that found experimentally up to pH 10.5; but above that it would have Curve *B*. This is also not the case.

The experimental data agree well with Curve *C* which corresponds to the values in Column 5 of Table I. *This supports the theory that the basic group in proteins having a titration index at pH 3.8 to 4.6* (depending on the protein) *is due to a group which yields arginine on hydrolysis* and may be properly called *prearginine.*

III

EXPERIMENTAL

The two experiments, recorded in Tables II and III, were performed in water-jacketed hydrogen electrode cells[6] with a saturated KCl liquid junction. A separate solution was made up, from the mother solution, for each determination. The cells were standardized against 0.100 M HCl (pH 1.075).

The four experiments in Tables IV to VII were performed with considerably higher concentrations of edestin (as high as 14 per cent) and it was necessary to use a titrating electrode with agar-KCl junction. In each experiment all the readings were taken on a single solution to which molar NaOH (or HCl) was added with a weighing burette.

Owing to the large correction for hydroxyl ion concentration to be made above pH 13, it was not found satisfactory to standardize the electrodes against HCl. Hence the electrodes were standardized against 0.05 M NaOH before (and sometimes after) each experiment. In Fig. 2 are the values of pH $-$ log *oh* for NaOH[7] as determined with the water-jacketed (liquid junction) cells. By using this chart as a standard of pH $-$ log *oh* in correcting the edestin data, any errors due to the standard cell, the sodium hydroxide solution or the condition of the electrodes, would be eliminated in the calculation of $\frac{h - oh}{c}$.

[6] Simms, H. S., *J. Am. Chem. Soc.*, 1923, **45**, 2503.

[7] *oh* represents hydroxyl ion *concentration* in moles per liter. The value of pH $-$ log *oh* is equal to $pK_W + \log f_{OH}$ where f_{OH} is the activity coefficient of the hydroxyl ion (namely, $f_{OH} \times oh = OH$ = hydroxyl ion *activity*).

This method does assume, however, that solutions of the same ionic strength have the same liquid function potential and dielectric constant. The ionic strength of the protein solutions will be discussed below.

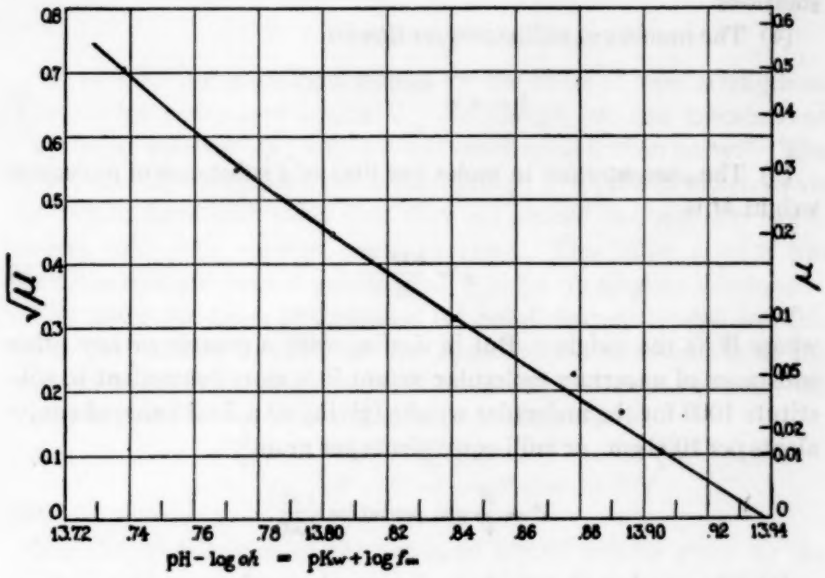

FIG. 2. *Data of the titration of NaOH.* Assuming complete dissociation, the *concentration* of hydroxyl ion (*oh*) is taken equal to the molarity of NaOH.[7]

IV

Calculations

A. Equivalents of Combined Base

The data in Tables II to VII were calculated as follows:

(1) The volumes of *strong base* (V_B) or *strong acid* (V_A) were calculated from the weight of the standard molar solutions, using the density at 25°C.

(2) The volume of *solution* (V) was taken as the sum of the volumes of water plus strong base (plus strong acid when present) and represents *protein-free* volume.

(3) The number of *millimoles* of strong base *minus* acid is:

$$1000\ (B - A) = 1000\ (V_B\ M_B - V_A\ M_A) \tag{1}$$

where M_B and M_A are the moles per liter in the standard base and acid solutions.

(4) The number of millimoles *per liter* is:

$$b - a = \frac{1000\ (B - A)}{V} \tag{2}$$

(5) The *concentration* in moles per liter of a substance of molecular weight M is:

$$c = \frac{1000\ W}{M\ V} \tag{3}$$

where W is the weight. But in dealing with a protein or any other substance of uncertain molecular weight it is more convenient to substitute 1000 for the molecular weight (giving us a final value of equivalents per 1000 gm., or milli-equivalents per gram):

$$c' = \frac{W}{V} \text{ gm. per ml.} = \frac{c\ M}{1000} \tag{3a}$$

(6) The number of *equivalents* of strong base minus acid is:

$$\frac{b - a}{c} = \frac{M\ (B - A)}{W} \text{ equivalents added per mole} \tag{4}$$

Or, for a protein:

$$\frac{b - a}{c'} = \frac{1000\ (B - A)}{W} \text{ equivalents added per 1000 gm.} \tag{4a}$$

(7) The ionic strength (μ) was calculated as explained below and used with the curve in Fig. 2 to obtain the value of pH $-$ log oh. The experimental pH value was subtracted from this and the value of $\frac{h - oh}{c'}$ was calculated (where h may be neglected in alkaline solutions).

(8) The "corrected equivalents of base," or the "combined base," is:

$$b' = \frac{b - a}{c} + \frac{h - oh}{c} \text{ equivalents combined per mole} \tag{5}$$

Or, for a protein:

$$b'' = \frac{b - a}{c'} + \frac{h - oh}{c'} \text{ equivalents combined per 1000 gm.} \qquad (5a)$$

$$b'' = b' \text{ when } M = 1000$$

B. Calculation of Ionic Strength

If we take the experimental data for the effect of ionic strength on protein ionization and assume a point charge, we can calculate an "apparent valence" (ν_A) which is not much greater than unity.[8] This is because the charges on the ionized groups in a protein molecule are so distant from each other that they act almost like separate monovalent ions (*i.e.*, *separate* point charges). The value $\nu_A = 1.8$ was found in acid solutions of gelatin, and $\nu_A = 2.4$ in alkaline solutions.

By using the same assumptions (of point charge) we can use this apparent valence to calculate the approximate contribution of the protein to the ionic strength of its solution. In alkaline solution the contribution of the protein and its bound cation will be:

$$\mu_0 = \frac{\nu_A^2 + \nu_A}{2\nu_A} b'' c' = \frac{\nu_A + 1}{2} b'' c' = 1.7\, b'' c'$$

while the contribution of the unbound NaOH will be given by the hydroxyl ion *concentration*, hence:

$$\mu = 1.7\, b'' c' + oh + a$$

$$= 1.7\,(b - a - oh) + oh + a$$

$$= 1.7\, b - 0.7\, oh - 0.7\, a$$

The data of edestin were calculated in this manner and confirmed the findings with gelatin that the contribution of protein to the ionic strength is even less than that given by this approximation. The results in Tables II to VII and Fig. 1 were calculated by assuming $\nu_A = 1$ (*i.e.*, infinitely distant point charges), hence:

$$\mu = b'' c' + oh + a = b$$

(where the amount of strong base b, being greater than the strong acid a, includes all free inorganic salt which may be present).

[8] Simms, H. S., *J. Gen. Physiol.*, 1928, **11**, 613.

TABLE II

Titration of edestin in jacketed electrodes with saturated KCl liquid junction at 25°C. (pH standard: 0.100 M HCl has pH 1.075).

Each solution contained 0.025 gm. edestin, plus 0.015 millimole of NaOH, plus the indicated volume of 0.0200 M HCl and was made up to 10.0 ml. $c' = 0.0025$ and $\mu = 0.0015$. All the solutions were cloudy.

V_A	pH	$\frac{b-a}{c'}$	b''
0.40	9.848	0.280	0.243
.50	9.135	.200	.193
.60	8.181	.120	.119
.70	7.993	.040	.039

TABLE III

Titration of edestin in jacketed electrodes with saturated KCl liquid junction at 25°C. (pH standard: 0.100 M HCl has pH = 1.075).

Each solution contained 0.0625 gm. edestin plus 0.0375 millimole of NaOH, plus the indicated volume of 0.0200 M HCl and was made up to 10.0 ml. $c' = 0.00625$ and $\mu = 0.00375$ in each solution.

V_A	pH	$\frac{b-a}{c'}$	b''
0	10.886	0.600	0.431
0.25	10.710	.520	.408
.50	10.482	.440	.374
.75	10.208	.360	.325
.87	10.078	.320	.294
1.00	9.898	.280	.263*
1.12	9.396	.240	.235*
1.25	9.195	.200	.196*

* Cloudy.

TABLE IV

Titration of edestin in a titrating electrode with agar-KCl bridge at 25°C. (pH standard: 0.0494 M NaOH has pH = 12.562).

4.996 gm. (W) of edestin in an initial non-protein volume of 35.00 ml. containing 2.420 millimole NaOH. The amount of 0.991 M NaOH is indicated by V_B (determined by weight; d = 1.040 at 25°C.).

V_B	$c' = \frac{W}{V}$	μ	pH	$\frac{b-a}{c'}$	b''
2.442	0.1428	0.069	11.438	0.484	0.457
2.774	.1414	.078	11.672	.550	.503
2.960	.1407	.083	11.807	.587	.523
3.246	.1396	.090	11.979	.644	.547
3.859	.1372	.105	12.216	.765	.591
4.457	.1350	.119	12.372	.884	.627

TABLE V

Titration of edestin in a titrating electrode with agar-KCl bridge at 25°C. (pH standard: 0.0494 M NaOH has pH = 12.562).

2.500 gm. (W) of edestin in an initial non-protein volume of 17.72 ml. containing 1.708 millimole NaOH. The volume of 0.991 M NaOH is indicated by V_B (determined by weight; d = 1.040 at 25°C.).

V_B	$c' = \frac{W}{V}$	μ	pH	$\frac{b-a}{c'}$	b''
1.724	0.1411	0.0964	11.966	0.683	0.569
5.523	.1161	.254	13.040	2.19	0.65
8.619	.1015	.346	13.217	3.41	.72

TABLE VI

Titration of edestin in a titrating electrode with agar-KCl bridge at 25°C. (pH standard: 0.0494 M NaOH has pH = 12.562).

2.500 gm. (W) of edestin in an initial non-protein volume of 18.14 ml. containing 2.168 millimole NaOH. The volume of 0.991 M NaOH is indicated by V_B (determined by weight; d = 1.040 at 25°C.).

V_B	$c' = \frac{W}{V}$	μ	pH	$\frac{b-a}{c'}$	b''
2.188	0.1371	0.109	12.376	0.867	0.615
4.583	.1218	.221	12.936	1.817	.692

TABLE VII

Titration of edestin in a titrating electrode with agar-KCl bridge at 25°C. (pH standard: 0.0492 M NaOH has pH = 12.560).

0.600 gm. (W) of edestin in an initial non-protein volume of 30.45 ml. containing 0.413 millimole NaOH. To this was added 0.991 M HCl in volumes indicated by V_A (determined by weight; d = 1.016 at 25°C.).

V_A	$c' = \frac{W}{V}$	μ	pH	$\frac{b-a}{c'}$	b''
0	0.01971	0.0136	11.423	0.688	0.518
0.072	.01967	.0159	11.144	.570	.481
.139	.01962	.0180	10.841	.458	.414
.196	.01957	.0198	10.383	.365	.349
.230	.01956	.0209	10.083	.308	.300*

* The last solution was cloudy. The other solutions were clear.

V

SUMMARY

The author corroborates the data of Schmidt showing that the dissociation index of the third group of arginine is $pK_3' = 12.5$.

New titration data of edestin have been obtained in very alkaline solutions and show that there is a corresponding group with a titration index of $pG' = 12.0$, but present in much less quantity than can account for the arginine found on hydrolysis. The data support the theory that the combination of strong base or strong acid with proteins is produced by the formation of salts with the "extra groups" of those trivalent amino acids which can be isolated from the protein, with the exception of arginine. Arginine contributes to the titration curve in much smaller amount than is found on hydrolysis. This deficiency in the arginine group may be accounted for by the basic group in proteins having a titration index of $pG' = 3.8$ to 4.6 (depending on the protein), which apparently yields arginine on hydrolysis, and may properly be called *prearginine*.

THE COMBINATION OF EDESTIN WITH HYDROCHLORIC ACID

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(Accepted for publication, July 7, 1930)

In a recent paper[1] it was shown that the amounts of the ions of HCl combined with gelatin could be calculated from electromotive force measurements of cells without liquid junction, of the type Ag, AgCl, HCl + protein, H_2. The maximum combining capacity of gelatin for H^+, as calculated by this method, agreed with values obtained in other ways. The present paper records experiments in which this method has been applied to the protein edestin.

EXPERIMENTS[2]

A sample of edestin had been prepared from ground hemp seed by Osborne's method,[3] slightly modified.[4] It was freed from electrolytes by dialysis against running distilled water in a rocking apparatus of the type described by Kunitz and Simms.[5] The liquid obtained by centrifuging the suspension after dialysis had a specific conductivity of 4.4×10^{-5} reciprocal ohms at 30°C., while the corresponding value for the water used was 2.6×10^{-6}. Solutions were prepared by mixing portions of the suspension with measured amounts of standard hydrochloric acid, made up from a distilled constant boiling mixture and redistilled water, and checked volumetrically by Na_2CO_3 and gravimetrically by AgCl. The concentrations of acid and of edestin were determined by weighing the whole solution containing a known volume of standard acid, neutralizing a weighed portion to methyl red with NaOH, and drying this neutralized portion to constant weight at 110°C. The weight of edestin was obtained by subtracting the weight of NaCl (calculated from the original HCl content of the solution) from the observed dry weight. The weight of water in a given weight of solution was obtained by sub-

[1] Hitchcock, D. I., *J. Gen. Physiol.*, 1928-29, **12**, 495.

[2] Most of the measurements were made by Esther R. Mason. The edestin was prepared by C. E. Heinrichs.

[3] Osborne, T. B., *J. Am. Chem. Soc.*, 1902, **24**, 28.

[4] Hitchcock, D. I., *J. Gen. Physiol.*, 1921-22, **4**, 597.

[5] Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1927-28, **11**, 641.

tracting the sum of the weights of edestin and HCl from the total weight. The concentrations were expressed as molality of HCl and grams of protein per 1000 gm. H_2O. The measurements of E.M.F. were carried out at 30°C., as described in the previous paper.[1] The data are given in the first 3 columns of Table I. Each figure for the E.M.F. represents the mean of results with 3 or 4 cells not differing by more than 0.0003 volt, and each constant to ±0.0001 volt for at least 1 hour.

TABLE I

Electromotive Force at 30°C. of the Cells

Ag, AgCl, HCl, H_2 and Ag, AgCl, HCl + edestin, H_2

m	g	E (observed)	E'_0	E (calculated)	ΔE	pH (approx.)
0.0100	0	0.4650	0.2244			
0.0200	0	0.4303	0.2260			
0.0301	0	0.4101	0.2271			
0.0401	0	0.3958	0.2278			
0.0501	0	0.3848	0.2284			
0.1004	0	0.3510	0.2309			
0.1007	13.05	0.3568	0.2307	0.3570	+0.0002	1.08
0.1020	18.55	0.3592	0.2307	0.3592	0	1.11
0.1050	42.35	0.3732	0.2304	0.3730	−0.0002	1.32
0.1055	62.70	0.3961	0.2301	0.3961	0	1.67

m = moles HCl per 1000 gm. H_2O.
g = gm. protein per 1000 gm. H_2O.
E (observed) = E.M.F. in volts, corrected to 1 atmosphere dry H_2.
$E'_0 = E + 0.1203 \log m$, for cells containing HCl alone.
E (calculated) $= E'_0 - 0.06015 \log (m - gx)(m - gy)$.
x = moles H^+ combined with 1 gm. edestin = 13.4×10^{-4}.
y = moles Cl^- combined with 1 gm. edestin = 3.9×10^{-4}.
$\Delta E = E$ (calculated) $- E$ (observed).
pH (approx.) $= -\log (m - gx)$.

Interpretation of Results

The agreement of the values in the third and fifth columns of Table I indicates that the data may be explained by assigning to 1 gm. of edestin a combining capacity of 13.4×10^{-4} equivalents of H^+ and 3.9×10^{-4} equivalents of Cl^-, both figures being constant in the range of these experiments, from about pH 1.1 to 1.7. The assumptions used in arriving at these values, and the method of calculation, are as fol-

lows. The relation between E.M.F. and ionic activity is given by the thermodynamic equation,

$$E = E_o - 0.06015 \log m_H m_{Cl}\gamma^2, \quad (1)$$

where E is the observed E.M.F. in volts, E_o is a constant, m_H and m_{Cl} are the molalities of the ions, and γ is the geometric mean activity coefficient of H^+ and Cl^-. Letting $E'_o = E_o - 0.1203 \log \gamma$, this becomes $E = E'_o - 0.06015 \log m_H m_{Cl}$, or

$$E = E'_o - 0.06015 \log (m - gx)(m - gy). \quad (2)$$

Here m is the total molality of HCl, x and y are the numbers of moles of H^+ and Cl^-, respectively, combined with 1 gm. of protein, and g is the number of grams of protein per 1000 gm. H_2O in the solution. As a first approximation, the value of E'_o for the protein cells was assumed to be constant and equal to that calculated for the cell containing 0.1004 M HCl without protein by the relation

$$E'_o = E + 0.1203 \log m, \quad (3)$$

which follows from equation (1) and the definition of E'_o. When the equations obtained by substituting the measured values of E, m, and g in equation (2) were solved simultaneously for x and y by the graphical method of the previous paper,[1] the curves did not intersect in a single point, but the provisional values $x = 13.4 \times 10^{-4}$ and $y = 3.5 \times 10^{-4}$ were obtained by averaging the different intersections. This relatively high value for y indicated a possible error in the assumption that E'_o (or γ) was constant for all the solutions. If all the protein is combined with H^+, and a certain amount also combined with Cl^-, then the latter part should behave as un-ionized molecules. Hence the ionic strength of such a solution should not be equal to m, but to $m - gy$. Provisional values of $m - gy$ were calculated and values for E'_o corresponding to them were interpolated from a plot of the E'_o values given for pure HCl in the first part of Table I. These values for E'_o are given in the last part of Table I, and were used in re-calculating the edestin data. A second application of the graphical method again failed to give a single intersection of the curves for y as a function of x, but the values selected from this plot, $x = 13.4 \times 10^{-4}$ and $y = 3.9$

$\times 10^{-4}$, gave such agreement of the observed and calculated values for E that further repetitions of the procedure seemed unnecessary. The second approximation reduced the average deviation of the calculated values (average of $\triangle E$) only from 0.00013 to 0.00010 volt.[6]

DISCUSSION

The value here obtained for the combining capacity of edestin for H^+ is slightly higher than any of the values compiled by Cohn,[7] who selected 12.7×10^{-4} moles HCl per gram edestin as the probable maximum value. In an earlier paper[4] curves were given by the writer which lead to the value $12.7 (\pm 0.6) \times 10^{-4}$ equivalents of HCl or H_2SO_4, and the same number of moles of $(COOH)_2$ or H_3PO_4, as the combining capacity of 1 gm. of edestin at pH 2.0 or below. Somewhat later[8] another curve was obtained for HCl which points to the value 14.7×10^{-4}. These divergent values are probably to be explained by insufficient removal of combined acid or base from some of the protein preparations. Attempts to purify edestin are handicapped by the uncertainty which still exists as to the exact location of its isoelectric point. It should be added that, according to Osborne,[3] edestin is changed by acid solutions into a substance insoluble in NaCl solutions. Hence any value for the maximum combining capacity with acids is probably a property of this denatured substance edestan rather than of the native protein edestin.

The present value, like all previous values obtained from experiments with edestin in solution in hydrochloric acid, is much lower than that recently reported by Bancroft and Barnett[9] for the combining capacity of dry edestin with hydrogen chloride gas. Their figure of 110 mg. or 30.2×10^{-4} equivalents HCl per gram edestin is more than twice the present figure of 13.4×10^{-4}. The latter falls

[6] A recalculation by this second approximation method of the values given for gelatin (Ref. 1) did not change them significantly. The difference between the values for E'_0 for 0.1 M HCl in Table I and in the previous paper is probably due to differences in the AgCl electrodes, which were prepared by the electrolytic method and used repeatedly without replating.

[7] Cohn, E. J., *Physiol. Rev.*, 1925, **5**, 349.

[8] Hitchcock, D. I., *J. Gen. Physiol.*, 1922–23, **5**, 383.

[9] Bancroft, W. D., and Barnett, C. E., *J. Phys. Chem.*, 1930, **34**, 449.

on the horizontal part of their curve of pressure of HCl gas against weight of HCl taken up. If the present figure, which appears to result from the saturation with H^+ of certain groups of the protein in solution, had any significance in their experiments, it might be expected to show up as a step in their curve, such as they found with gliadin and HCl. The absence of any such break in their curve seems to indicate that the protein behaves quite differently in solution and in the dry state. Both figures cannot represent the maximum combining capacity of edestin for HCl, and it is quite possible that neither does. Further experiments would be required to determine whether the protein is more altered by solution in 0.1 M hydrochloric acid or by saturation with dry hydrogen chloride gas. The fact that in the present experiments the E.M.F. remained constant for some hours would seem to indicate that, if there is any alteration in the protein, it takes place quickly and the system reaches a steady state. The discoloration of the dry protein which they report may be indicative of some secondary chemical reaction between the protein and the gas, which may possibly open up other acid-combining groups not sharing in the reaction in solution.

SUMMARY

Electromotive force measurements of cells without liquid junction, of the type Ag, AgCl, HCl + protein, H_2, lead to the conclusion that 1 gm. of edestin (or, more probably, edestan) combines with a maximum of 13.4×10^{-4} equivalents of H^+ and 3.9×10^{-4} equivalents of Cl^-, when the protein is dissolved in 0.1 M HCl.

THE INTRINSIC RHYTHM OF THE TURTLE'S HEART STUDIED WITH A NEW TYPE OF CHRONOGRAPH, TOGETHER WITH THE EFFECTS OF SOME DRUGS AND HORMONES

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It is well known, since the time of Ringer, that an excised heart, if properly supplied with oxygen in a physiological salt solution, will beat for several days, in fact until the tissue begins to disintegrate. Snyder[1] in connection with his studies on the effect of temperature on the heart beat of the Pacific terrapin (*Chemmys marmorata*) counted rates at intervals over periods of 11 hours and observed considerable changes in rate under fairly constant conditions. At 25°±1°C. the heart of the terrapin did not beat longer than 6 hours, due probably to the fact that this species is adapted to cold water.

The question arises as to the constancy with which a heart can beat over a period of many hours or several days, if all external factors affecting rate are kept quite uniform. For this type of investigation some sort of *continuous automatic* recording mechanism is a necessity. The Loomis chronograph, a preliminary description of which has already been published,[2] is specially designed to record rates of periodically recurring processes.

Anything which will actuate a relay will run the chronograph whose mechanism is essentially as follows:

The time for ten pulsations to occur is recorded by the length of a line of ink drawn by a moving pen on a revolving sheet of paper. During the next four pulsa-

[1] Snyder, C. D., *Univ. Calif. Pub. Physiol.*, 1905, **2**, 125.

[2] Loomis, A. L., and Harvey, E. N., *Science*, 1929, **70**, 559.

tions the pen is returned to its zero position and is then ready to record the average time for ten more pulsations. Each line therefore represents fourteen pulsations and tells the average time for ten pulsations to occur. The machine is driven by a synchronous motor running on the A.C. lighting circuit. By reducing gears and pulleys the paper is revolved at the proper speed and the pen, attached to a pulley string, is drawn across at a constant rate of ten units per second. The string pulley which draws the pen is actuated by an electro-magnetic clutch in connection with a fourteen toothed wheel and an escapement, also worked magnetically. Each pulsation to be recorded is made to open an electric circuit that magnetizes the electro-magnet moving the toothed wheel one tooth. While the wheel revolves ten teeth the magnet clutch moves the pen at constant speed across the paper, and during the next four teeth the magnetic clutch is out and a spring pulls the pen back to the first position. If ten pulsations occur in 10 seconds the unit lines on the paper (ten to a second) represent a 1 per cent difference in rate and the paper can be calibrated by recording pulses of current at a rate of one per second. By mere inspection of the paper record one can observe any change in rate and very quickly calculate the percentage constancy of the rhythmic process.

Later models of the chronograph have been modified by (1) introducing an easily changeable gear box for driving the chronograph at different speeds; (2) returning the pen to its 0 position by revolving pulley wheel instead of by a spring, thereby shortening the recovery to two beats instead of four; (3) replacing the toothed wheel and escapement with an improved type of rachet mechanism that cannot miss a beat; (4) placing a small electro-magnet in connection with the moving pen so that it is lifted off the paper with each beat, recording not only the average time for ten beats, but the interval between beats as well; (5) introducing a device for recording the time between single pulses or series of pulses without missing the time interval involved in the return of the pen to its zero position.

A complete description of the mechanical details of the chronograph will be published elsewhere.

In its new form the chronograph will record rates of 300 per minute to 1 beat in 6 minutes with absolute fidelity, giving a record which shows at a glance the change of rate without counting each individual beat. Figs. 2 and 3 give an idea of the records that can be made. The swing of a pendulum, manual beating of time, pulsation of a tambour, the fall of drops from a tube, the flashing of neon lamps, or the speed of rotation of any device can all be recorded by the proper electrical contact or relay mechanism. The growth of plants can also be recorded. It is only necessary to arrange a contact device of such a nature that, when the growing tip of the plant makes contact with a surface, the surface is automatically lifted a definite distance. A certain time interval will elapse before the plant grows sufficiently to make another contact. By knowing distance and time interval, which the chronograph records, we can determine (and record) the rate of growth. A continuous record of changes in light intensity can also be obtained by a very simple accessory mechanism.

The heart offers the best known case of a biological rhythm whose rate change is of fundamental importance. Action currents of the human heart or of animals picked up by the cardiotachometer of Boas[3] or by any properly designed amplifying device make beautiful records. For recording the fundamental heart rhythm we wished to avoid cardioaccelerator and cardio-inhibitory effects, and the following procedure, illustrated in Fig. 1, was followed.

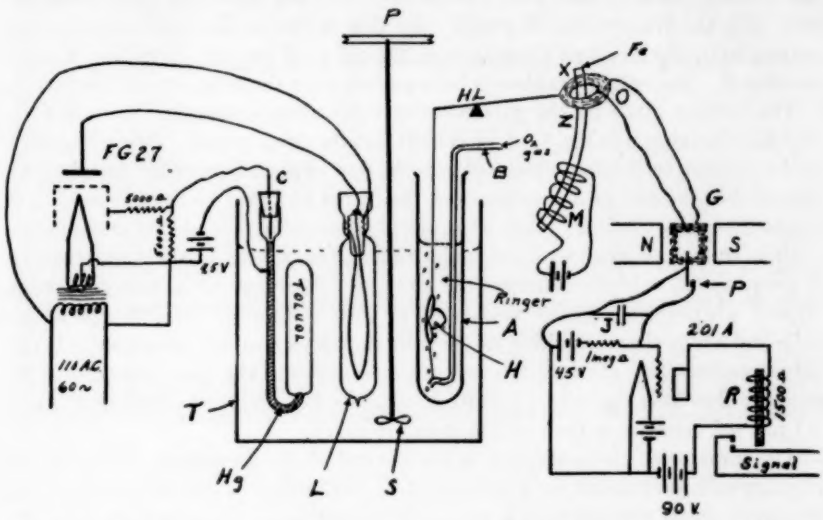

FIG. 1. Diagram of heart in thermostat showing wiring connections to chronograph. Explanation in text.

The heart of a turtle [either the painted (*Chrysemys picta*) or the slider (*Pseudyms rubriventris*) were used, and showed no characteristic differences] was removed, taking no special care to keep sinus intact, and mounted in a tube (*A*) of Ringer's solution[4] by tying threads to the tip of each auricle, one thread being wound around the end of an L-shaped glass tube (*B*) through which oxygen was bubbled into the Ringer, the other attached to the short arm of a heart lever (*HL*). It would seem that the easiest method of making an electrical contact would be through the long arm of the heart lever dipping into a pool of mercury. However, as is well known,

[3] Boas, E. P., *Arch. Int. Med.*, 1928, **41**, 403; Boas, E. P., and Weiss, *J. A. M. A.*, 1929, **92**, 2162.

[4] The Ringer's solution contained NaCl, 0.65 per cent; KCl, 0.014 per cent; $CaCl_2$, 0.014 per cent; $NaHCO_3$, 0.02 per cent; and sometimes glucose, 0.2 per cent.

the turtle's auricle contains smooth muscle fibers whose periodic contraction causes tone changes in the auricles which are often *greater than the heart muscle contractions*, and are, of course, indicated by a change in position of the heart lever. This means that at times the lever arm will never leave the pool of mercury, and at other times the heart will never relax enough for the lever to touch the mercury surface, consequently spoiling the record and involving continual adjustment. To obviate this difficulty, a coil of fine wire (*O*) was attached to the end of the heart lever which moved the coil over the end of an electro-magnet (*M*), thus cutting the field of force and inducing a small current in *O*, sufficient to actuate a galvanometer relay (*G*), the Weston No. 30 model. By this device we always induce a small current in *O*, whether tone changes cause the coil to be moving in position *X* or in position *Z*. Recording has always been perfect after the adoption of this device.

The contact made by the galvanometer relay actuates another vacuum tube (201A) relay, shown in Fig. 1, which is built into the chronograph. The advantage of the vacuum tube relay, which controls the flow of plate current by charging the potential of the grid of a vacuum tube, lies in the fact that no current need flow across the contact points. Only a change of potential occurs and slight oxidation of these contact points does not introduce enough resistance[5] to affect the working of the relay (*R*), a high resistance (1500Ω) type. The magnet is actuated by plate current whenever a negative voltage of 45 volts is removed from the grid of the tube (201A), by short circuiting the 45 volts through a megohm resistance. If the galvanometer relay contacts are very short in duration the plate current can be made to flow long enough to actuate relay *R* by introducing a condenser (*J*) of 0.1 to 1 mf. capacity in the position shown in Fig. 1.

The same principle is adopted in the control of the thermostat (*T*) with the mercury-toluol regulator, the heating lamp (*L*) and stirrer *S*, with the pulley drive, *P*, except that a vacuum tube is used, a thyratron[6] (FG27) whose plate circuit is supplied by the 110 A.C. lighting mains with which the heating lamp is in series. The filament circuit is supplied by a 110 to 5 volt transformer and a negative grid voltage of 4½ volts is applied whenever the mercury rises in the regulator and makes contact with the contact wire, *C*. This cuts off the plate flow and puts out the heating lamp which will light again as soon as the contact is broken and 4½ volts negative removed from the grid. The outfit is an ideal regulating device for thermostats.

The temperature control of our hearts was 25°C. +0.02°C.; oxygen[7] was continually bubbled through at a constant rate; and the Ringer's solution was buffered so that constant pH was attained. As the heart was excised, cardio-motor reflex disturbances were eliminated. Everything worked automatically so that no

[5] The contact can be made through the body of an individual.

[6] We are deeply indebted to the General Electric Company for this thyratron.

[7] In some experiments air was used instead of oxygen, but no difference in the rhythmic behavior of the heart was noted.

mechanical disturbances accompanying adjustment could have had an influence on the rate recorded, which we believe represents the fundamental intrinsic rhythm of some periodic process within the heart muscle cells. Twenty-five hearts in all have been studied at 25°C., some of them recorded for as long as 36 hours.

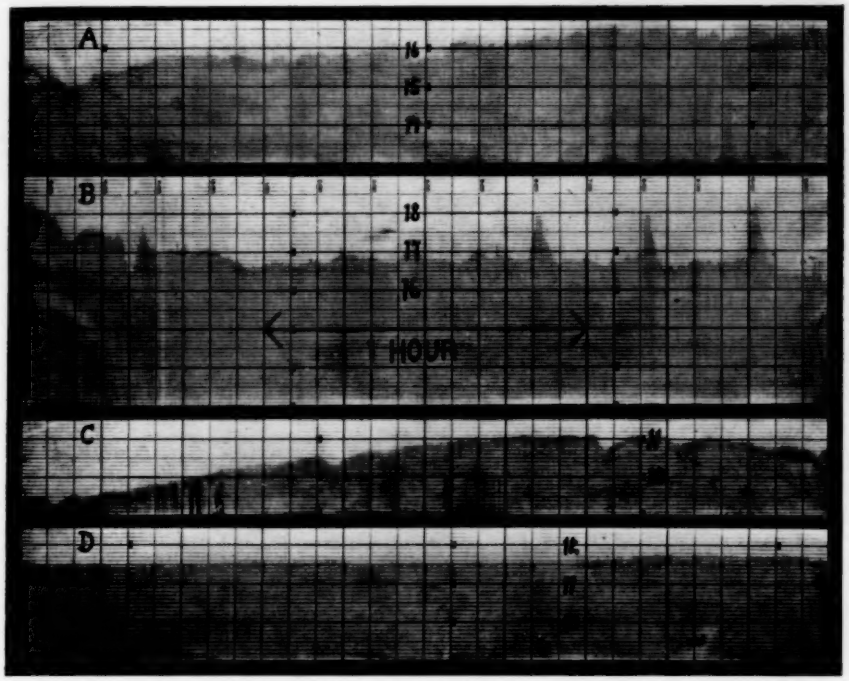

FIG. 2. Portions (2½ hours) of records of rate of turtle's heart in Ringer's solution without glucose under constant conditions at 25° ±0.02°C. *A*. Sept. 23, 1929, 4 hours after start, showing considerable rhythmic changes in rate. *B*. Same heart after 8 hours, showing marked rhythmic decreases in rate exactly 20 minutes apart. *C*. Sept. 22, 1929, 40 minutes after start, showing rhythmic increases in rate exactly 50 minutes apart. *D*. Same heart, 4 hours later, showing remarkably constant rate. Ordinates, seconds for ten beats. Abscissae, 15 minute periods. Each vertical line represents fourteen beats of the heart and records the average time for ten beats to occur. The longer the ordinate line the slower the heart.

There are several important observations which have come from these records. (1) A considerable variation in the behavior of hearts from different turtles (Fig. 4). The rate is by no means constant over

a long time period. Neither does the rate continually decrease, although the general trend is in that direction. Usually the rate decreases during the first hour or two, but the rate may be the same at the

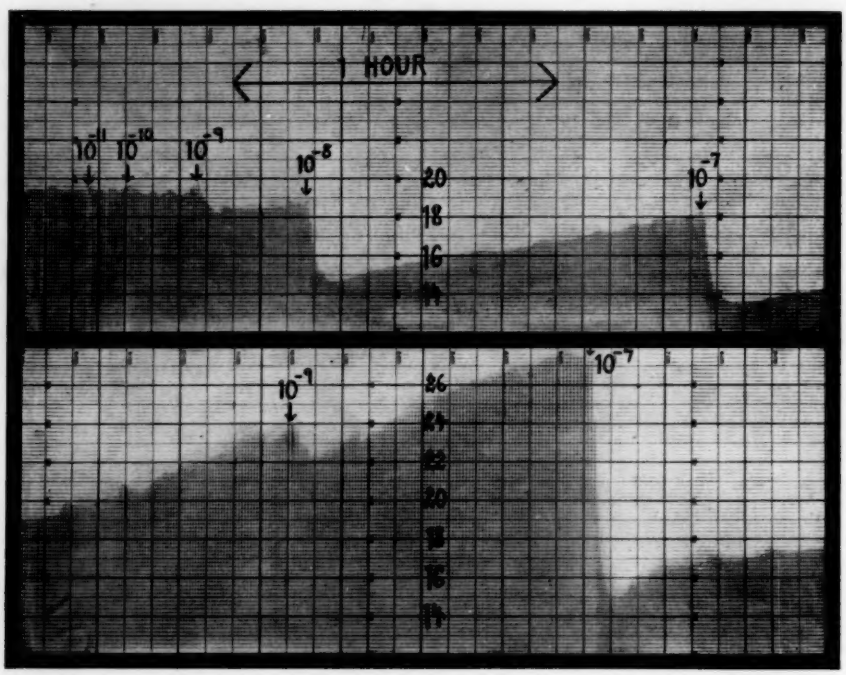

FIG. 3. Portions of a 32 hour record of the rate of a turtle's heart in Ringer's solution + 0.2 per cent glucose under constant conditions at 25° ±0.02°C. Above—Fairly constant rate with slight rhythmic changes 3 hours after start, showing effect of adding increasing concentrations of adrenalin. Note first effect in increasing rate occurs with 10^{-9} gm. adrenalin per cubic centimeter and marked effect with 10^{-8} adrenalin. Below—Same heart with adrenalin effect gradually wearing off 90 minutes after end of *A* portion. Note again detectable increase in rate from adrenalin in 10^{-9} gm. per cubic centimeter and very marked effect of 10^{-7} adrenalin on a slowly beating heart. Abscissae, 15 minute periods. Ordinates, seconds for ten beats. The longer the ordinate line the slower the heart.

end of 12 hours, having made some fluctuations in the meantime. This initial decrease in rate may be connected with the seeping of adrenalin out of the tissue or the oxidation of adrenalin in the tissue. The ampli-

tude of contraction always decreases and is very weak when the heart is about ready to stop. Often beats will be omitted under these conditions, that is, there will be two beats and one omitted, or three beats and one omitted, etc. The greatest variation in rate noted was about 33 per cent in a 5 hour period and 60 per cent in a 12 hour period, and

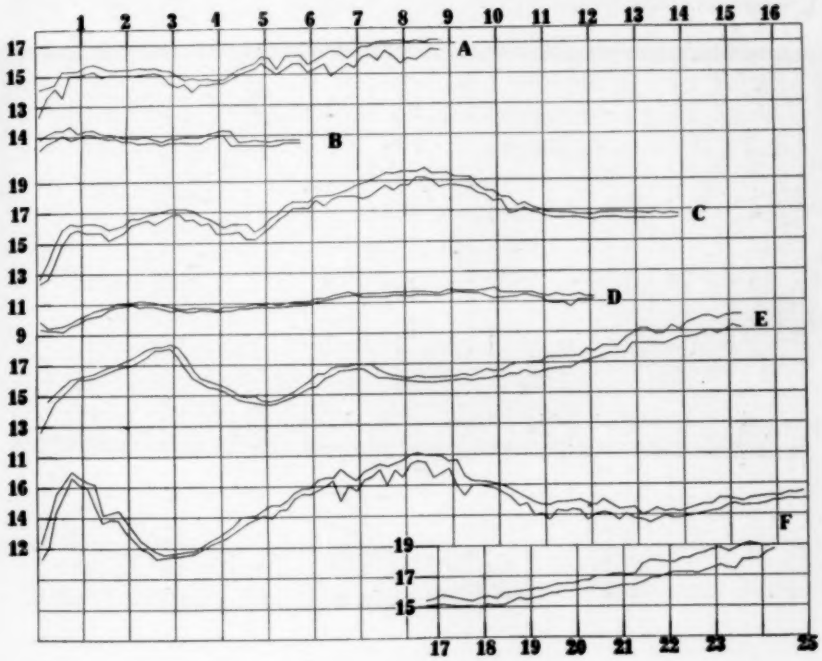

FIG. 4. Plots of rates of six hearts (*A* to *F*) Ordinates, seconds for ten beats. Abscissae, hours after heart removed and mounted in Ringer's solution at 25° ±0.02°C. All conditions constant, no drugs or hormones added. *A*. June 16, 1929. *B*. June 14, 1929. *C*. June 17, 1929. *D*. Sept. 22, 1929. *E*. Oct. 7, 1929. *F*. Oct. 6, 1929. Abscissae for latter part of this record at bottom of chart. The curves were drawn by plotting the slowest rate (upper line) and the most rapid rate (lower line) for each 10 minute period.

the least variation was 2 per cent in a 5 hour period and 1 per cent over half hour periods (Fig. 2, *D*). This is quite remarkable regularity as it means that a heart will beat 100 times with a change of only one beat.

(2) The occurrence of rhythms in rate of beat which often recur at

rather definite intervals, that is periodic changes in the rate. A heart may show a 6 per cent decrease in rate lasting 3 minutes every 20 minutes for four 20 minute periods (Fig. 2, *B*), or similar 3 to 4 per cent increases in rate lasting a few minutes for three periods 50 minutes apart (Fig. 2, *C*). Such rhythmic changes in rate are not connected with the tone changes often observed in the auricles, whose

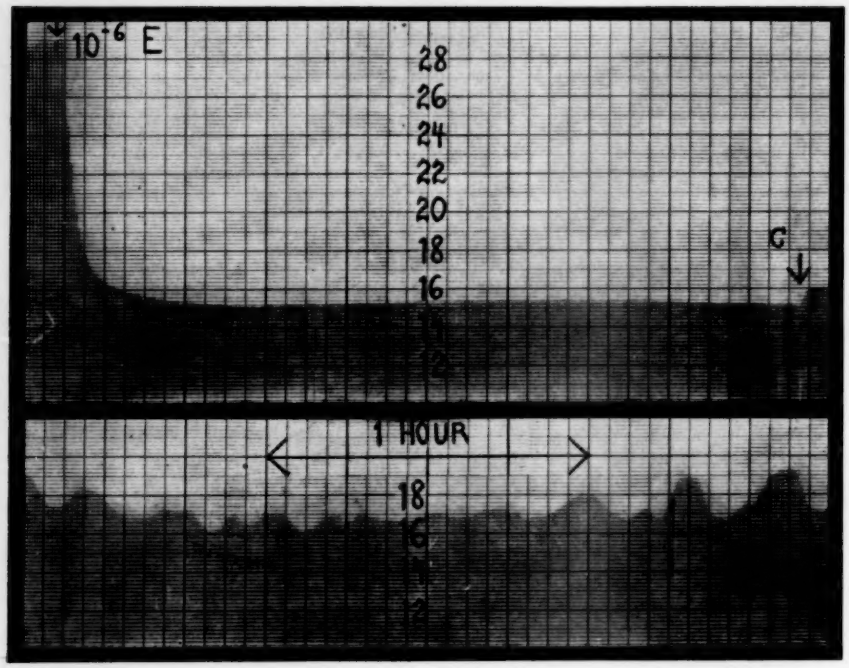

FIG. 5. Portions (2½ hours) of records of rate of turtle's heart showing: Above—effect of epehedrin (1:10^6) at *E* and effect of chloretone (1:20000) at *C*. Note remarkable regularity after ephedrin. Below—normal heart showing marked rhythmic changes in rate. Ordinates, seconds for ten beats. Abscissae, 15 minute periods. The longer the ordinate line the slower the heart.

period is 40 to 50 seconds, nor can they be traced to any change in the environment. They are true variations of the fundamental rhythm. Most frequently the maximum of these rhythms occur at intervals of 5, 6, or 10 minutes, but the period of such superposed rhythms is continually changing (Fig. 5).

These rhythmic changes are shown by practically all the hearts. Thinking that they might be connected with spontaneous activity of the postganglionic inhibitory nerve cells in the heart we tried adding atropin in concentrations of $1:10^6$, $1:10^5$, and $1:10^4$ to the Ringer's solution. These concentrations should paralyze the inhibitory nerve endings, but our records show no effect on the rhythmic changes in rate. The high concentration caused a progressive slowing of the rate.

Adrenalin or ephedrin added to the Ringer's does abolish the rhythmic rate changes, at the same time causing the well known increase in rate. One part of adrenalin in 10^9 parts of Ringer's causes a perceptible increase in rate (about 8 per cent) and $1:10^8$ adrenalin causes a 21 per cent increase followed by a very regular and progressive slowing presumably due to slow oxidation of the adrenalin (Fig. 3). During this progressive slowing the heart beats with remarkable regularity, that is, no indication of periodic changes in rate. The percentage increase in rate after adding adrenalin is greater if the heart is beating slowly when the adrenalin is added than it is when the heart is beating more rapidly. These effects are illustrated in Fig. 3. To keep a heart in good rhythmic condition over long periods of time our observations suggest that a slight concentration of adrenalin should be present in the medium bathing the hearts.

As is well known ephedrin has a physiological effect which is similar to adrenalin. Our records show that $1:10^6$ ephedrin added to Ringer's solution may double the rate of a slowly beating heart (10 beats in 30 seconds). The increase in rate takes 25 minutes for completion and the effect lasts for hours. Fig. 5 shows an ephedrin effect on a slowly beating heart. Note the difference in the form of the effect as compared with adrenalin. For greatest constancy in rate, a little ephedrin should be added to the Ringer's solution.

(3) Another characteristic of some hearts is a *sudden* change of rate. Both sudden increases in rate (15 per cent) and sudden decreases in rate (13 per cent) have been observed. They presumably indicate a sudden dropping out of one pace-making region or the sudden appearance of another. We cannot connect them with any changes in the environment.

α and β pituitary hormone[8] had no effect on the rate when freed of the chloretone which is added to preserve these solutions. The chloretone slows the rate slightly as shown in Fig. 5. The β hormone (pitressin) contained 10 pressor units per cubic centimeter and the α hormone (pitocin) contained 10 oxytocic units per cubic centimeter. 2 cc. of each one was added to 200 cc. Ringer's solution in testing. Insulin (Eli Lilly Company), 20 units per cubic centimeter also had no effect on the rate when 2 cc. were added to 200 cc. Ringer's solution.

Our results with thyroxin[8] were somewhat variable. In six experiments using $1:10^5$ thyroxin dissolved in a minimal amount of alkali, the increase in rate ranged from 0 to 26 per cent. The increased rate appears fairly quickly, but not as rapidly as with adrenalin, and is prolonged. It is possible that increased alkalinity of the Ringer's had some influence on the heart, as this factor was not sufficiently controlled.

Nicotin ($1:10^4$) caused a progressive slowing, as did ergotamine tartrate ($1:10^5$). Adrenalin administered after ergotamine showed the well known slowing of rate instead of an increase. Our records show that this chronograph is admirably adapted for studying the effects of drugs.

In conclusion we wish to express our thanks to Mr. Charles Butt, Technical Assistant in Physiology, Princeton University, for help in taking many of the records.

SUMMARY

A chronograph is described for recording continuously the rates of many different kinds of rhythmic processes over long time periods. The rate is read directly from the length of a line of ink, drawn by a moving pen.

Rates of beat of excised turtle's hearts in Ringer's solution have been recorded at 25°C. under constant conditions of temperature, pH,

[8] We are deeply indebted to Parke Davis and Company for a supply of α and β pituitary hormones, and to E. R. Squibb and Sons for 100 mg. of crystalline thyroxine.

and oxygen supply for periods of 36 hours. Regular periodic variations in the fundamental rhythm are figured, as well as rates of extraordinary constancy. The effects of adrenalin, ephedrin, thyroxin, α and β pituitary hormone insulin, nicotin, and atropin are described in the text.

THE ACCUMULATION OF ELECTROLYTES

I. The Entrance of Ammonia into Valonia macrophysa

By WM. C. COOPER, Jr., and W. J. V. OSTERHOUT

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Experiments on the penetration of NH_3 into *Valonia macrophysa*, carried out in Bermuda in 1924–27, are described in the present paper. The interpretation of these results will receive further discussion in subsequent articles.

In order to ensure uniformity of material and of sea water a large number of cells, collected at the same time, were kept under the same conditions and all the sea water used for these cells and for the experiments was collected at the start and kept in carboys. The average volume of the cells was about 0.5 cc.

The experiments were carried out in stoppered bottles each containing 2400 cc. of solution with about 50 cells in each bottle. When the bottles were placed in the ocean they were fastened beneath a specially constructed raft so that the light received was mostly reflected from the whitish coral sand of the bottom. In the experiments in the laboratory direct sunlight was avoided. Growth and absorption of ammonia were more rapid in the ocean.

The temperature in the ocean varied from 20° to 25°C., but in the course of any one experiment the variation was not more than 2° or 3°C., except in those lasting several weeks. In experiments carried on in the laboratory the variation was much greater.

The NH_4Cl was added to the sea water in the form of a solution, 0.6 molar or normal in NH_4Cl; the pH value of the sea water was adjusted by the addition of HCl or NaOH. After exposure to the sea water containing NH_4Cl, the sap was collected by means of a pipette having a sharp point which was thrust into the cell (in order to avoid loss of NH_3 by suction, the sap was forced into the pipette with the fingers). Care was taken in collecting the sap to use some large and some small cells so that the sap[1] could be considered as taken from an average cell of about 0.5 cc. When sufficient sap had been extracted, it was blown out into a small beaker and covered. Two 5-cc. samples (for zero point determinations there were three) were pipetted out for sodium-potassium determinations, two 1-cc. samples for

[1] For analysis of the sap see Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, **5**, 225.

ammonia, two 1-cc. samples for halide, and a single 1-cc. sample for pH and sulfate. The samples for sodium-potassium analysis were stored in quartz, the others in pyrex vessels. The samples for ammonia determination were at once acidified to prevent escape of NH_3.

Analysis of the various samples was made according to standard procedures: potassium was determined as potassium perchlorate (accurate with quartz vessels, to within 0.1 per cent): sodium by indirect analysis as sodium chloride (accurate to within 1 per cent); ammonia (total) by colorimetric determination as $NHg_2I \cdot H_2O$ after distillation of the ammonia from the strongly alkaline solution and subsequent Nesslerization (distillation was necessary because the turbid condition of the Nesslerized raw sap made color estimation exceedingly difficult); halide was determined by the Volhard method; the pH values were determined colorimetrically, allowing for the salt error by adding NaCl to the standard buffer solutions.

For the volume measurements, each cell was carefully dried with cloth and filter paper and weighed rapidly to milligrams, then placed in 0.005 M NH_4Cl sea water in a specific gravity bottle which was then immersed in a thermostat and allowed to come to a temperature of 25°C. The bottle was then dried and weighed to milligrams. From these weighings the cell volume was calculated. Such cells were photographed in groups (under water) to facilitate future identification, the volume of each being noted on the photograph.

When NH_4Cl is added to the sea water it soon appears inside the cell,[2] where its concentration may become over 40 times as great as the sea water, thereby lessening the specific gravity of the sap[3] so much that the cells, leaving their normal situation on the bottom, rise to the surface, where they continue to grow. The change in the specific gravity of the sap is shown in Fig. 1.[4]

Experiments were made to determine the rate of penetration at

[2] For experiments on the penetration of NH_3 into *Valonia* see Brooks, M. M., *Pub. Health Reports*, Washington, D. C., 1923, **38**, 2074; for experiments on other cells see Warburg, O., *Z. physiol. Chem.*, 1920, **66**, 305; Harvey, E. N., Carnegie Institution Publication, 1914, *No.* **183**, p. 131; Irwin, M., *J. Gen. Physiol.*, 1925–26, **9**, 235; Jacobs, M. H., Harvey Lectures, 1926–27, p. 146.

[3] *Cf.* Cooper, W. C., Jr., Dorcas, M. J., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1928–29, **12**, 427. Osterhout, W. J. V., Some fundamental problems of cellular physiology, New Haven, 1927. This curve is based on experiments in the laboratory.

[4] The change is more rapid when the cells, enclosed in bottles, are placed in the ocean; this may be due in part to light (*cf.* Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1923–24, **6**, 47; Hoagland, D. R., Hibbard, P. L., and Davis, A. R., *J. Gen. Physiol.*, 1926–27, **10**, 121.

various pH values. Since the initial rate is most easily interpreted the experiments were limited to 10 minutes which is as short a period as is practicable.

In each bottle containing 120 cc. of solution were placed 6 cells (each cell containing about 0.2 cc. of sap). Cells were selected as far as possible of the same size and shape. The bottles were placed on a slowly revolving wheel to keep the contents stirred. Ordinarily this treatment produced no injury which could be

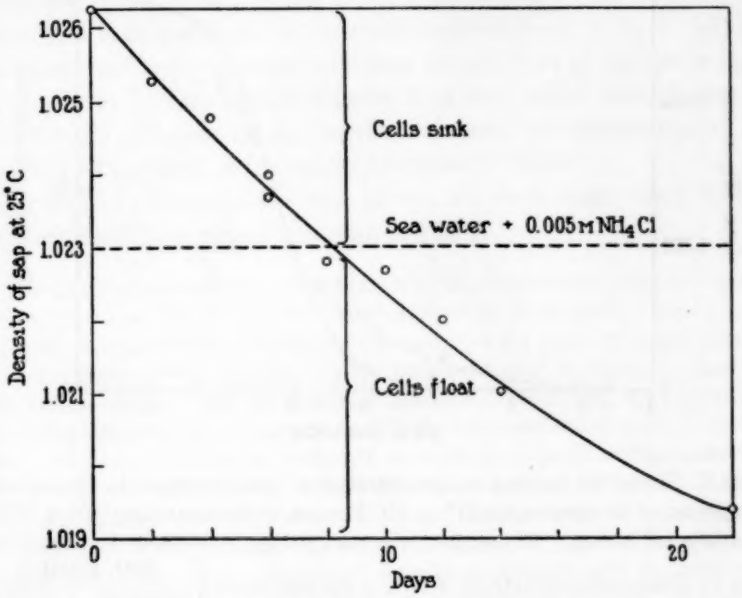

FIG. 1. Time curves showing the decrease in the specific gravity of the sap during the penetration of ammonia from sea water (at pH 8.3) containing 0.005 M NH_4Cl.

detected during the experiment or by subsequent observation (during several days) of cells kept in sea water. The ammonia (*i.e.*, $NH_3 + NH_4OH + NH_4^+$) of the sap of untreated cells (about 0.0006 M) was subtracted from the ammonia values of the sap of treated cells to give the values shown in Fig. 2. The pH value of the sea water was adjusted by adding NaOH or HCl; the total ammonia of the sea water was kept constant.

The results are shown in Fig. 2 and it is evident that as the concentration of the undissociated base (*i.e.*, $NH_3 + NH_4OH$) increases the

rate[5] of penetration of ammonia (total) also increases. But it is not easy to determine just how closely they agree. If we knew the rate of penetration when the total ammonia (*i.e.*, $NH_3 + NH_4OH + NH_4^+$) is present as the undissociated base ($NH_3 + NH_4OH$) it would be easy to see whether the rate is reduced to one half when the concentration of

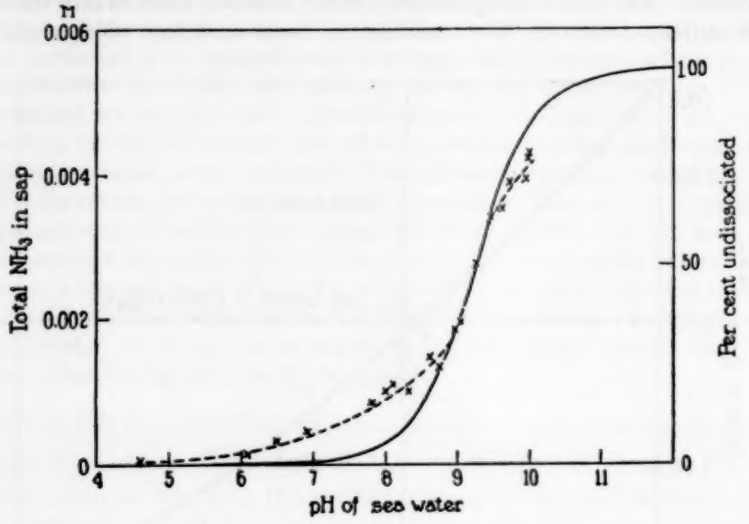

FIG. 2. Shows the increase in concentration of total ammonia in the sap after an exposure of 10 minutes (at 21° ± 1°C.) to sea water containing 0.01 M NH_4Cl at various pH values. Broken line, observed values; continuous line, calculated curve for dissociation of $NH_4OH + NH_3$ in distilled water $\left(\frac{(NH_4^+)\,(OH^-)}{(NH_4OH) + (NH_3)} = K_B = 10^{-4.74}\right)$. Since it happens that the inflection points of both curves lie at approximately pH 9.26 they have been made to coincide there: at that point the ordinate of the observed curve is 0.00275 M and this point is taken as 50 per cent of the calculated dissociation curve (whose inflection point and 50 per cent dissociation point coincide).

the undissociated base is reduced to one half, but unfortunately we cannot safely perform experiments at a sufficiently high pH value to ensure that practically all of the ammonia is present in undissociated form (even at pH 10.1, which is as high as we have gone, less

[5] Experiments with dead cells show little or no effect of pH value on penetration.

than 87 per cent of the total ammonia is regarded as present as the undissociated base).

It seems probable that in the absence of complicating factors the curve might resemble the equilibrium curves[6] for H_2S and CO_2, so that the inflection point would occur where the rate of entrance and the per cent of undissociated base was one half the maximum. We find that the point of inflection of the rate curve occurs at pH 9.25, which corresponds fairly well to the point (9.26) at which one half the total ammonia is in the form of the undissociated base ($NH_3 + NH_4OH$) as calculated from conductivity data of solutions of ammonia in distilled water.[7] This would suggest that the initial rate of entrance reaches 50 per cent of its maximum when the undissociated base reaches 50 per cent of its maximum concentration.

In order to compare the two curves we have made them coincide at their inflection points as shown in Fig. 2.

We should expect the point of inflection of the dissociation curve to occur at a somewhat higher pH value in sea water on account of the greater ionic strength. In order to determine approximately this value for sea water NH_3 was circulated in a closed glass vessel (designed by Dr. Grinnell Jones[8] to whom our thanks are due for allowing us to use his invention in advance of publication), containing on one side ammoniacal sea water and on the other, distilled water to which sufficient NaOH had been added to bring the pH value above 12 under which conditions all of the ammonia exists as the undissociated base ($NH_3 + NH_4OH$). The total ammonia was kept constant and the pH value of the sea water was varied by adding HCl or NaOH; after equilibrium had been attained at any given pH value a sample was taken of the ammoniacal water in equilibrium with the ammoniacal sea water and the ammonia determined by colorimetric comparison. We therefore made measurements which gave a curve indicating that the inflection point, *i.e.*, the point where half the total ammonia is in the form of the undissociated base, is in the neighborhood of pH 9.5.

If the rate of entrance of ammonia is proportional to the external concentration of the undissociated base we might expect the curves to coincide, but this could be true only if the internal pH value remained constant. If, for example, the pH of the sap were kept constant at 12

[6] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–28, **8**, 131; Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, **9**, 255.

[7] This gives $K_A = 10^{-14} - K_B = 9.26$ and $K_B = 4.74$.

[8] Jones, G., *J. Am. Chem. Soc.*, 1928, **50**, 1600.

so that the entering ammonia did not dissociate inside, there would be no complications, but with sap at lower pH values a part of the entering NH_3 would be transformed to ions and, therefore, the back pressure, or the tendency of the undissociated base to return to the sea water, would be diminished. In other words, the rate of penetration of ammonia would be a function not only of the external pH but of the internal pH as well.[9]

Our experiments show that the entrance of NH_3 raises the internal pH value rapidly (Fig. 3), this effect being greater the higher the concentration of undissociated ammonia in the sea water. Hence we expect the rate to be relatively higher at the lower pH values of the sea water because under these conditions there is a smaller penetration of ammonia and consequently less effect on the pH of the sap (normally about 5.8). This would increase the ordinates of the curve progressively toward the left[10] and consequently make the slope of the curve less steep, *i.e.*, like that of the observed curve.[11]

The fact that the initial rate of penetration increases when the concentration of undissociated molecules increases suggests that it is chiefly undissociated molecules which penetrate, but it has been pointed out elsewhere that this does not necessarily follow.[12] It does indicate that the penetrating substance is NH_3 or NH_4OH or the ion pair $NH_4^+ + OH^-$, rather than NH_4Cl whose rate of entrance would not be expected to increase as the external pH value increases.

That the substance penetrating at the start is largely NH_3 (or

[9] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 261.

[10] We should expect a similar effect if penetration depended on chemical combination of undissociated molecules with some constituent of the protoplasm (or on absorption), since the amount of the compound formed would determine the rate of penetration and this would increase more and more slowly as the external concentration of undissociated molecules increased.

[11] This can easily be seen by making the ordinates of the dissociation curve progressively larger as we go toward the left and then multiplying all the ordinates by the same factor so as to make the maximum ordinate the same as for the dissociation curve.

[12] This results from the fact that if the ion pair $NH_4^+ + OH^-$ enters, its penetration is proportional to the ionic activity product $({}^a NH_4) \times ({}^a OH) = K ({}^a NH_4OH)$, so that as the penetration of the indissociated base increases that of the ion pair will also increase. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 261.

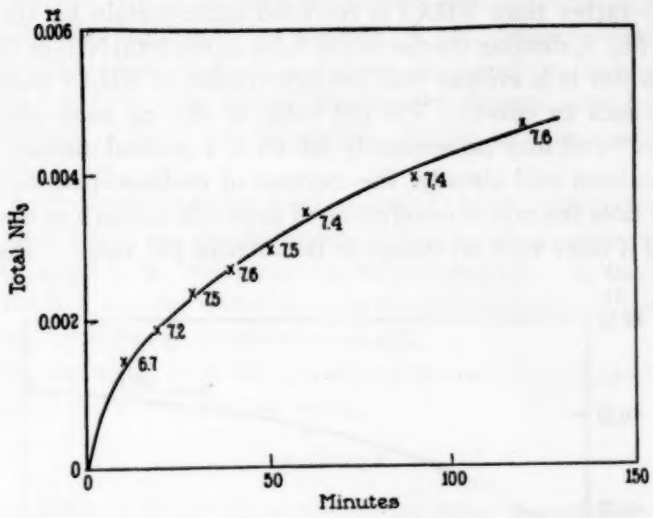

FIG. 3. Time curves showing the increase of pH value of sap during penetration of ammonia from sea water, at pH 8.1, containing 0.005 M NH_4Cl.

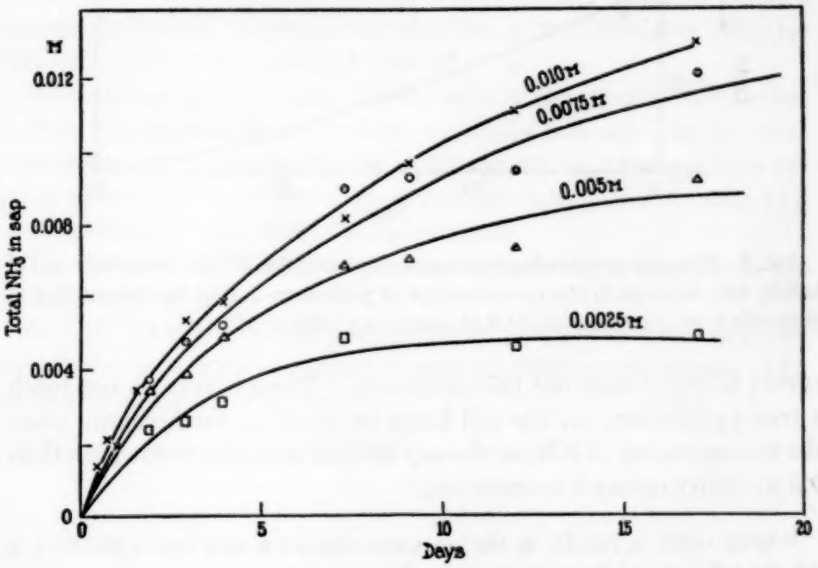

FIG. 4. Time curves showing increase of total ammonia during penetration from sea water (at pH 8.3) containing various amounts of NH_4Cl.

NH_4OH) rather than NH_4Cl is rendered quite certain by the data given in Fig. 3, showing the rise in pH value as the total NH_3 in the sap increases (for it is evident that the penetration of NH_4Cl would not produce such an effect). The pH value of the sap soon reaches a maximum[13] and may subsequently fall off in a gradual manner.

As has been said already, the increase of undissociated ammonia does not raise the rate of penetration of ammonia as much as would be expected if there were no change in the internal pH value. The time

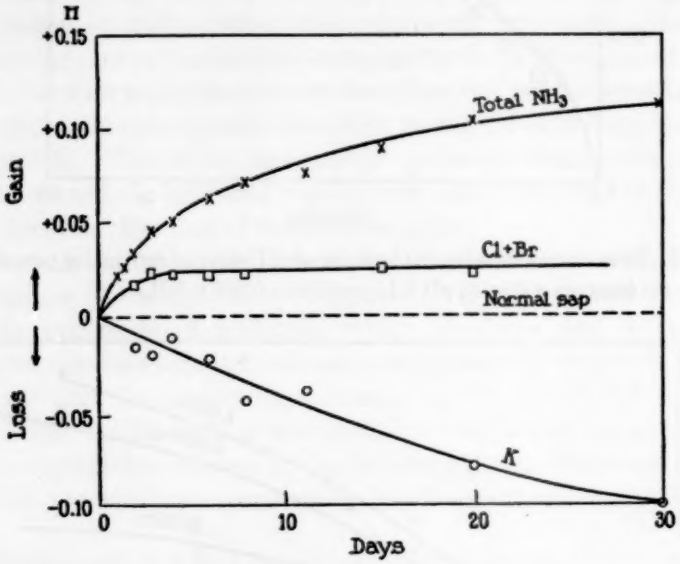

FIG. 5. Time curves showing increase in concentration of total ammonia and of halide, and decrease in the concentration of potassium during the penetration of ammonia from sea water (at pH 8.3) containing 0.005 M NH_4Cl.

curves in Fig. 4 bear out this statement. These curves do not reach a true equilibrium, for the cell keeps on growing; furthermore, when the concentration of NH_3 in the sap reaches a certain point (less than 0.3 M) injury causes it to come out.[14]

[13] With 0.001 M NH_4Cl in the sea water this is 6.6, with 0.01 M NH_4Cl it is 8.2, the pH value of the sea water being 8.0.

[14] *Cf.* Irwin, M., *J. Gen. Physiol.*, 1925–26, **9**, 235.

Fig. 5 shows that as the total ammonia in the sap increases, the concentration of potassium in the sap falls off, and measurements of volume[15] indicate that the decrease was not due merely to the absorption of water but that some potassium actually diffused out of the cell. The behavior of Na is not yet certain: further experimentation is in progress.

Fig. 5 also shows that the initial entrance of ammonia is accompanied by an increase in the concentration of halide, which soon reaches a fairly constant value dependent on the external pH. In the absence of ammonia in the sea water, there is no such variation in the halide of the sap with change in the external pH.

It may be added that the growth of the cells is increased by the addition of NH_4Cl to the sea water.

SUMMARY

When 0.005 M NH_4Cl is added to sea water containing cells of *Valonia macrophysa* ammonia soon appears in the sap and may reach a concentration inside over 40 times as great as outside. It appears to enter as undissociated NH_3 (or NH_4OH) and tends to reach a pseudoequilibrium in which the activity of undissociated NH_3 (or NH_4OH) is the same inside and outside.

When ammonia first enters, the pH value of the sap rapidly rises but it soon reaches a maximum and subsequently falls off. At the same time there is an increase of halide in the sap which, however, does not run a parallel course to the ammonia accumulation, but it comes to a new equilibrium value and remains constant.

The increase in NH_3 in the sap is accompanied by a decrease in the concentration of K.

As NH_3 enters the specific gravity of the sap decreases and the cells rise to the surface and continue to grow as floating organisms. The growth of the cells is increased.

[15] The cells grow faster when NH_3 is added to the sea water.

THE VARIATION OF ELECTRICAL RESISTANCE WITH APPLIED POTENTIAL

II. Thin Collodion Films

By L. R. BLINKS

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication, June 12, 1930)

I

Analogies to certain physiological phenomena have been observed in the behavior of collodion membranes. Collander,[1] Michaelis,[2] and Northrop[3] have reported resemblances in the penetration of certain substances; Michaelis[4] has shown the similarity of the potential differences of such membranes to those of apple skin and other cuticle; Labes and Zain[5] have imitated with collodion tubes such bioelectric effects as current of injury, polarizations in direct current, and the phenomena of electrotonus.

Furthering these analogies, the present paper amplifies some observations previously made[6] on very thin collodion membranes, which resemble the protoplasm of *Valonia* cells under some conditions[7] in

[1] Collander, R., *Soc. sci. Fennica, comment. biol.*, 1926, **2**, 1; *Kolloidchem.Beih.*, 1924, **19**, 72.

[2] Michaelis, L., and Weech, A. A., *J. Gen. Physiol.*, 1928–29, **12**, 55. See also Fujita, A., *Biochem. Z.*, 1926, **170**, 18.

[3] Northrop, J. H., *J. Gen. Physiol.*, 1927–28, **11**, 233; 1928–29, **12**, 435.

[4] Michaelis, L., *J. Gen. Physiol.*, 1925–28, **8**, 33; *Bull. National Res. Council*, 1929, No. 69, 119.

[5] Labes, R., and Zain, H., *Arch. exp. Path. u. Pharm.*, 1927, **125**, 29, 53; **126**, 284, 352.

[6] Blinks, L. R., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 359. Shortly after the publication of this preliminary note, Ebbecke (Physiological Congress, Boston, 1929) described similar effects with collodion, stating that the current across a membrane from HCl to salt was greater than from salt to HCl.

[7] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 793.

having an apparent resistance to direct current which varies with the voltage and the direction of an applied potential.

The conductance of collodion membranes was found by Michaelis[8] to vary greatly and characteristically with the solution of electrolyte in contact with them. His membranes, thoroughly imbibed by long washing, were usually in contact with the same solution on both sides. When they separated two different solutions (as in the experiments[9] where the transfer number of ions in the membrane was determined) there was at first a change of resistance with time, necessitating the adjustment of the current by a ballast resistance. This change was ascribed chiefly to polarization at the platinum electrodes.

In the present experiments, the most marked effects were found when the membranes separated two different solutions of different conductivity, such as HCl and KCl. The resistance then depended upon which ions were carried across the membrane, and the potential gradient under which they moved. It is believed that the changes are those of real ohmic resistance and that they present no phenomena requiring any special properties of collodion to explain, since the effects may be seen in other systems. The collodion film is thus used merely to obtain a very short conducting system separating two different solutions.

A rise of resistance always occurs when ions of lower mobility are moved into a conductor and supplant those of higher mobility. Devices for maintaining a constant current in a tube of electrolyte under these conditions are used in the determination of transport number by the "moving boundary" method.[10] A similar effect is more strikingly seen when a current passes through a fine capillary, filled with KCl solution and dipping into sea water; the resistance then rises when the positive current passes inward, and falls, when, it passes outward. This is an effect which must be controlled in the experiments performed with cells impaled on capillaries.[11]

[8] Michaelis, L., Green, A. A., and Weech, A. A., *J. Gen. Physiol.*, 1928–29, **12**, 473.

[9] Michaelis, L., Weech, A. A., and Yamatori, A., *J. Gen. Physiol.*, 1926–27, **10**, 685.

[10] Longsworth, L. G., and MacInnes, D. A., *J. Opt. Soc. Am. and Rev. Sci. Instr.*, 1929, **19**, 50.

[11] Blinks, L. R., *J. Gen. Physiol.*, 1930–31, **14**, 139.

In both the above cases the limits are represented by the resistance of the conductor when totally filled with one or the other solution. They tend to be reached at all current densities, the rise being slower with small voltages than with large. When sharp boundaries are maintained, this must be strictly true. But under very small potential gradients the boundaries become less sharp, and backward diffusion will prevent the complete substitution of one solution for another. (A heating effect may further contribute to this diffuse boundary at very high current densities.)

The most satisfactory explanation for the apparent equilibrium at any given potential in these collodion membranes is that a boundary of greater or less sharpness is formed, composed of different proportions of the ions involved. This maintains itself under the two opposed gradients of potential and diffusion, and as long as these remain constant, the character and position of the boundary is unchanged, giving a constant resistance. When either is changed the resistance changes. The process is exaggerated by the extreme thinness of the membrane, so that the boundary has but to move a short distance to the right or left in order to completely fill the membrane with one or the other electrolyte.

II

The membranes were prepared from ordinary Merck's collodion U. S. P.; or from Parlodion dissolved in 75 per cent alcohol, 25 per cent ether. The kind of collodion seemed to be immaterial for these effects. A small amount of the solution was poured upon a clean glass plate and another plate gently placed over it (avoiding bubbles), spreading the collodion into a film. The plates were then carefully slid apart and stood on end to dry. 15 minutes' drying was often sufficient for these very thin films, but usually they were allowed a day or more. They adhere very tightly to the glass and may be stored in this condition. Usually, however, they were removed from the glass and stored spread out on filter paper. This was done by immersing the glass plates in water until the films were free (loosened, if necessary, by a camel's hair brush) and floated to the top. They were then spread into a smooth sheet, and lifted out, supported on filter paper inserted from below. Dried flat they now remained slightly attached to the paper, and could be stored in folders until needed.

From regions which showed brilliant red and green interference colors 2 cm. squares of paper and film were cut with shears and the film cemented over the ends of tubes for use. These tubes were usually of the Y-form shown in Fig. 1. One

arm of the Y was gently heated and the end ringed with the low melting point wax "Picein" which adheres well to both glass and collodion. While still warm it was brought gently in contact with the square of collodion which formed a firm seal to the glass and came free of the filter paper. The other arm of the Y was filled with a plug of stiff agar gel, imbibed with the solution used in the Y-tube. The Y-tube was usually filled for a distance of about 1 cm. up the vertical neck. For emptying it was inverted and drained. Great care was needed to avoid vibration at all times as the thin membranes were easily broken by jar, even with the small head of liquid used.

The arms of the Y-tube dipped into intermediate vessels filled with the desired solutions as shown. The electrodes were large ones of chloride-coated amalgamated lead, and were practically free of polarization at any current density used in these experiments. No resistance changes of great magnitude were observed in

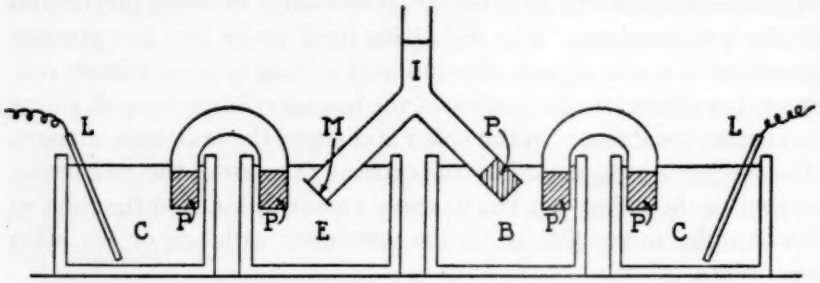

FIG. 1. Diagram of apparatus for measuring resistance of collodion films. The membrane (*M*) is cemented over one arm of the Y-tube, and separates the inner solution (*I*) from the outer solution (*E*). The other arm, plugged with agar (*P*), dips into the cup (*B*). U-tubes, plugged with agar (*P*), connect with the cups (*C*) into which dip the electrodes (*L*).

the system of conductors leading up to the membranes. With both arms of the Y-tube plugged with agar the resistance of the system under various conditions was between 500 and 1500 ohms; it remained constant to 50 or 100 ohms during long current flows under applied potentials of as much as 4 volts. This value formed the base which was subtracted from the total resistance to give that of the membrane. The resistance was measured in the direct current bridge previously described.[12]

The membranes introduced resistances from a few hundred ohms (when there were large leaks) to several megohms, as measured under standard conditions (in contact with 1.0 M KCl solutions). Neither of these extremes showed the phenomena of variable resistance as strikingly as the membranes of intermediate values most of which, as used, ranged between 3000 and 50,000 ohms, under standard

[12] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 361.

conditions. With care the solutions in contact with the membrane could be changed 8 or 10 times, and a return made to the first resistance value when in contact with the initial solutions. Sometimes a jar or the passage of a large current itself suddenly reduced the value of resistance under all conditions to a new level, at which the effects still took place, although often with a changed percentage. The resistance of the membranes had therefore to be checked frequently under standard conditions (in contact with 1 M KCl). When this was done a considerable series of comparisons could be instituted.

III

When a small potential was applied to a membrane separating two solutions, a definite resistance was soon reached, at which the bridge maintained a constant balance. When the potential was changed, a new value was produced, either higher or lower, depending upon the direction and intensity of the potential gradient: 15 seconds was usually sufficient to reach this new constant level, and this time was chosen as a standard in which to make the balance. In some cases a longer time was required, but in general the values at 15 seconds represented fully 95 per cent of the steady values.

The largest absolute changes of resistance were usually found in freshly made membranes immediately after being sealed to the tubes. As an example, such a new membrane had a net resistance of 9500 ohms when separating a solution of normal HCl inside the tube from sea water outside. This was the value when 50 mv. was applied to it, in such a direction that the positive current passed outward (from HCl toward sea water), moving H ions across the membrane. When the potential was increased in this direction the resistance fell further, reaching at 100 mv. 7800 ohms, and at 0.5 volt a value of 5200 ohms. At higher voltages the resistance became nearly constant at about 4800 ohms. On the return to a lower applied potential the resistance rose again and at 100 mv. was 7700 ohms. The potential was now reversed so that the positive current passed from sea water inward to HCl. At 50 mv. the resistance was 15,000 ohms; at increased voltages it rose rapidly, reaching at 0.5 volt 18,300 ohms, and at 3.3 volts 21,500 ohms. On the return to 100 mv. the value was 15,600 ohms.

After this membrane had been placed in sea water for 2 days, the resistance rose only to about 15,500 ohms at the maximum inward potential. A day later, after many measurements had been made, the

range was from 500 ohms (current outward) to 5800 ohms (current inward). There had evidently been progressive injury which increased the absolute permeability to ions. But the *percentage* of resistance rise under the same conditions had increased, probably because the current densities in the earlier experiments had not been sufficient for sweeping out all H ions from the membrane.

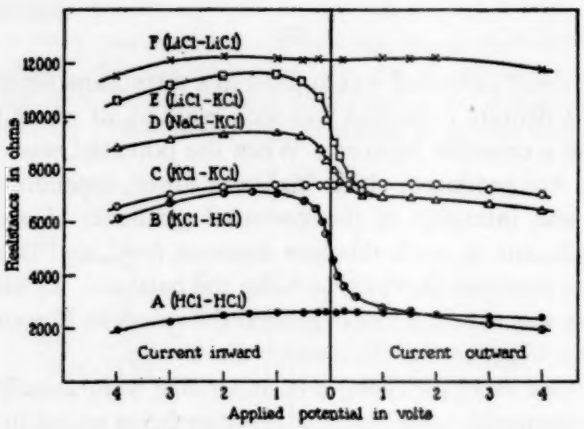

FIG. 2. Variation of resistance with applied potential in a membrane separating molar solutions, as follows:

Curve	*Outside solution*	*Inside solution*
A	HCl	HCl
B	KCl	HCl
C	KCl	KCl
D	NaCl	KCl
E	LiCl	KCl
F	LiCl	LiCl

Potentials increasing to the left of zero cause a flow of positive current *inward;* to the right *outward.*

This experiment may be considered analogous to many biological cases, where a cell encloses sap of high electrolyte concentration and is surrounded by water of low electrolyte concentration.

Even greater rises of resistance may be produced by greater dilutions of the sea water outside the membrane. But more useful information was gained by keeping the concentrations equal on both sides of the

membrane, and varying the *composition* of the solutions. In most of the experiments the concentration was kept at 1.0 molar, and chlorides only were employed, the cations being varied. Such a group of experiments is shown in Fig. 2 for HCl, KCl, NaCl, and LiCl. It is seen that the resistance increases in the order given. When two salts were separated by the membrane the resistance rose when the direction of the current was such as to carry the less mobile ions across the membrane. In general the highest value then reached (at high potentials)

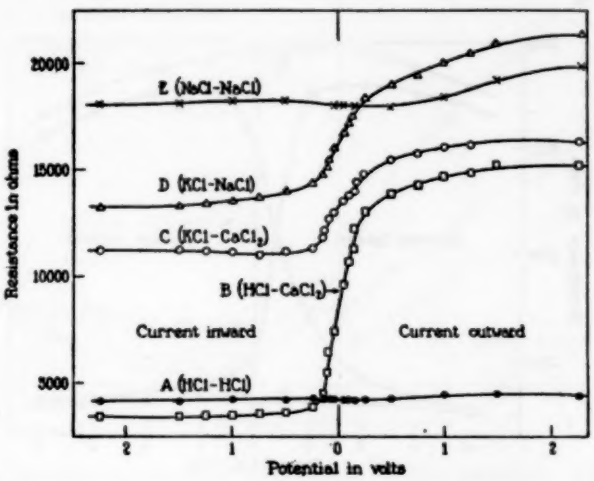

FIG. 3. Variation of resistance with applied potential in a membrane separating molar solutions of various kinds: Curve *A*, HCl both sides; *B*, HCl outside, $CaCl_2$ inside; *C*, KCl outside, $CaCl_2$ inside; *D*, KCl outside, NaCl inside; *E*, NaCl inside and outside. The membrane had evidently been somewhat injured between Curves *A* and *B*, and between *D* and *E*. Potentials increasing to left of zero cause inward current, to right, outward.

was a little lower than that found (at all potentials) when the membrane was exposed to the more poorly conducting solution on both sides. This showed that there was still some mixing of ions in the membrane at the highest potentials. The resistances of the membranes, when wholly imbibed with the given solutions, were about in the proportions of the specific resistances of aqueous solutions of the equivalent concentration. The membranes thus did not show any specific properties in exaggerating differences in ionic mobilities, such

as Michaelis found in his celloidin (Schering) membranes. The different kind of collodion, the higher salt concentrations used, and the thinness of the membranes may all account for the absence of this effect here. The membrane in 1 M LiCl had about twice the resistance it had in 1 M KCl, and in the latter had about three times that in 1 M HCl. The value with NaCl fell between KCl and LiCl, while in 1 M $CaCl_2$ (Fig. 3) it had a slightly lower resistance than in 1 M NaCl. These are roughly proportional to the specific resistances of the solutions.

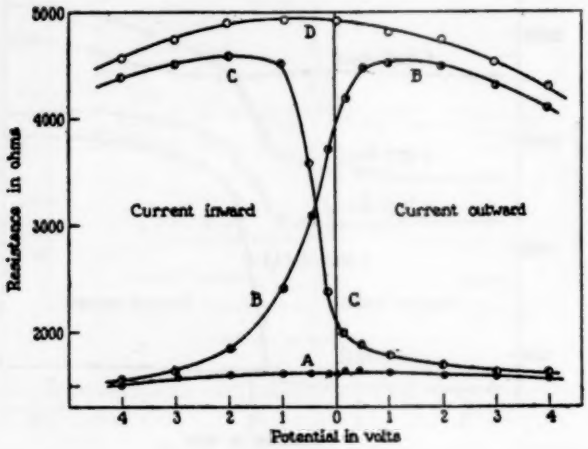

FIG. 4. Variation of resistance with applied potential in a membrane separating molar solutions of HCl and KCl. Curve *A*, HCl on both sides; *B*, HCl outside, KCl inside; *C*, KCl outside, HCl inside; *D*, KCl on both sides. Potentials increasing to left of zero cause inward current, to right, outward.

The alternating current resistance of these membranes was usually equal to that of direct current just where the curves crossed the line of zero potential.

While the resistances are in general dependent on the solutions with which the membranes are in contact, and hence are reversible when the solutions are changed side for side, there are also slight, and sometimes very large, asymmetries, which greatly distort the curves. Fig. 4 shows the values reached when KCl and HCl were applied to a membrane in four different combinations. With HCl inside the tube

and KCl outside the curve crosses the zero line at quite a different point from where it does with the opposite arrangement. The two curves also cross each other at a point about 300 mv. from the zero line.

It is also to be noted that the curve D is distinctly rounded, with the highest resistances at the lowest potentials. This was often found to be the case in the membranes, and may be due to a widening of cur-

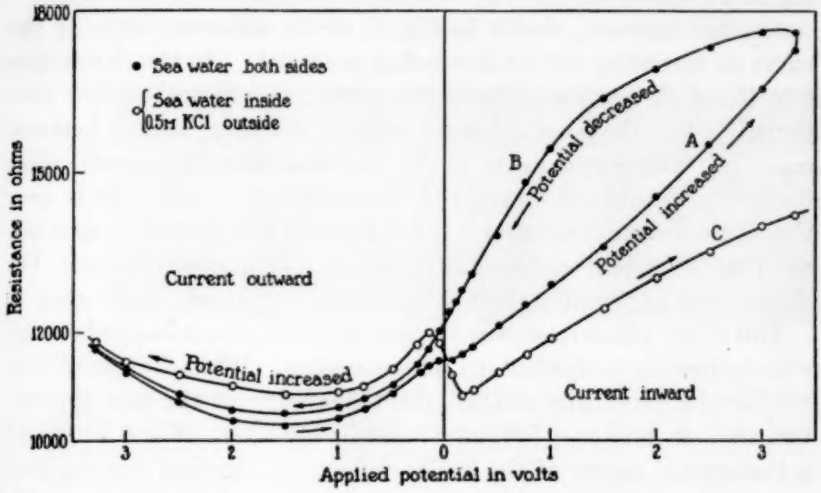

FIG. 5. Anomalies in the variation of resistance with potential. Curves *A* and *B* are for a membrane with sea water on both sides. Curve *A:* potentials decreased from left to zero, then reversed and increased from zero to right as shown by arrows. Curve *B:* potentials decreased from right to zero, while the current continued to flow. There is a hysteretic effect, the resistance remaining higher than during the increase of potential. To left of zero, however, with increase of potential during current flow, Curve *B* agrees well with Curve *A*. Curve *C* was taken after 0.5 M KCl had been substituted for sea water outside the membrane. The curve goes through a double inflection near the zero line. Potentials to left of zero cause *outward* current, to right, *inward*.

rent paths under high potentials. But the opposite shape, with the resistance *rising* on both sides of zero was occasionally found, and is so far without explanation. Fig. 5 shows a case of this kind, and also illustrates two other anomalies. It is evident that the resistance rise is not symmetrical about zero potential, being mostly confined to an *inward* current, decreasing at first with *outward* currents, and finally

rising again at higher potentials. When 0.5 M KCl was substituted for sea water on the outside of the membrane the curve went through a double inflection at the lower potential values. The remainder of the curve paralleled the sea water curve at slightly different absolute values. This double inflection was characteristic of several membranes just after solutions had been changed and is undoubtedly due to the establishment of new diffusion gradients in the membrane.

Another anomaly, shown in Fig. 5, is the different shape of the curve on ascending and on descending potentials. In the descending arm (*B*) of the sea water curve, the current was allowed to flow continually while the potential was decreased, not being broken between readings. These were made in the standard time (15 seconds) after the new potential was established across the membranes. It is seen that the resistance stayed at a much higher value at most potentials, and then began to decrease more rapidly, at the approach to zero. On the reversal of potential the original values were nearly duplicated.

This delay, which resembles a hysteretic effect, is seen in another way when potentials are applied in rapid succession. While the same resistance level is eventually reached, this takes longer at the first application than at later ones following immediately after. Fig. 6 is a string galvanometer record of the bridge deflections, showing two applications of the same potential to a membrane separating solutions of different conductivity. In this case the less mobile ions were being carried into the membrane by the current and the resistance rose during the flow of current. But the time to rise approximately to balance point (at break) was nearly 14 seconds, while the same point was reached in about 7 seconds on a new application, following 1 second after the first was broken. It is evident that during the 1 second between potential applications much diffusion had occurred, which had reduced the resistance again, and unbalanced the bridge; but this had not gone as far as in the original condition, and it did not take as long to establish the new gradient in the membrane. Very similar hysteretic effects have been noted in *Valonia* cells,[7] and the curves have much resemblance.

Comparison of this record with those of living cells does, however, show a striking difference as respects the back E.M.F. of polarization. When present this appears on the string records as a large upward excursion of the image at the removal of an applied potential. In

Nitella[13] and in normal *Valonia* this upward curve at "break" is nearly symmetrical with the downward movement at "make:" in this it resembles the curves for electrode polarization. But the upward movement at break is nearly absent in the records for collodion, showing that the rise of resistance is one of "real" or ohmic resistance.

In this respect these collodion membranes are in agreement with those of Espermüller[14] which were held only between metal plates, without any contact with solutions. The ions were there necessarily

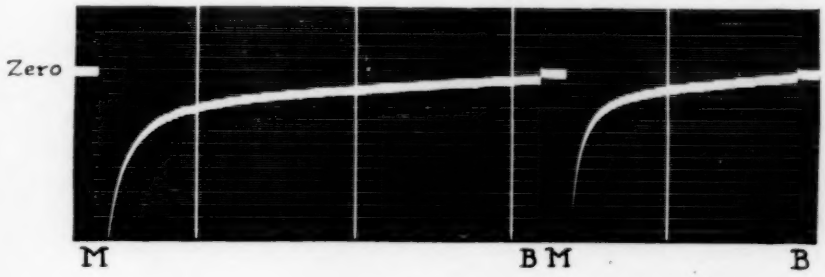

FIG. 6. String galvanometer record of bridge deflections during two applications of 0.5 volt to a membrane separating 1.0 M KCl from 1.0 M HCl. (Positive current from KCl to HCl.) The image deflects from zero (no current in bridge) downward at the application or "make" (*M*) of the potential and rises as the resistance increases nearly to the balance point. At removal of the potential or "break" (*B*) there is a negligible back E.M.F. displayed as the image returns to zero. At the second make (*M*) the deflection is less and the resistance rise is about twice as fast. At break (*B*), there is again a negligible back E.M.F. Time marks, 5 seconds.

derived from the collodion itself, and the resistance rose or fell as they were carried to one side or the other of the film. Polarization was absent.

IV

The biological significance of the phenomena described for collodion will depend upon the relative rôles played in living cells by the two components of any hindrance to the passage of direct current. These may be (1) a real ohmic resistance and (2) an E.M.F. of polarization.

[13] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 495.
[14] Espermüller, E., *Arch. Elektrotech.*, 1928, **21**, 148.

Both may occur in any conducting system and certainly the first is always present (with a possibility of variation). It would be possible to ascribe many of the observed resistance variations in living matter indirectly to variations in the bioelectric potential difference existing at the cell surface, which, by a small change of value (as at or near the zero point in these curves), might bring about a large change in the electrolyte content of that surface. This would affect not only direct current measurements, but also those of low frequency alternating current in which the ohmic resistance of the membrane is more important than its capacity. A tempting hypothesis could thus be established on the relations between bioelectric potential and electrical resistance.

The evidence, however, for *Valonia* and *Nitella*, as well as for many other organisms, indicates the greater importance of polarization E.M.F.'s in the observed "resistance" to direct current. The study of impaled *Valonia* cells[11] especially indicates the important rôle of these back E.M.F.'s. It is felt therefore that the mechanism of rise of resistance in the thin collodion films reported here does not completely represent that of the cells. It is, however, described since it may have a bearing upon other cases. It should further be noted that to a certain extent collodion furnishes a model for polarization potentials, inasmuch as Labes and Zain[5] found back E.M.F.'s of as high as 80 mv. developed by long passage of current through another type of membrane. Further study of these as a model of the *Valonia* cells is in progress.

SUMMARY

The resistance of very thin collodion membranes to direct current bears some resemblance to that of living cells since it varies with the applied potential. With membranes separating two different solutions the resistance varies with the direction (and the voltage) of the applied potential, rising when less mobile ions are carried across the membrane and falling when more mobile ones are so carried. (With some membranes the resistance varies with potential when the same solution is on both sides.) These changes are very prompt and regular. There is a hysteretic effect of previous current flow.

But the membranes differ from *Valonia* cells in that the rise of resistance is largely ohmic, there being little or no polarization potential.

THE VARIATION OF ELECTRICAL RESISTANCE WITH APPLIED POTENTIAL

III. Impaled Valonia ventricosa

By L. R. BLINKS

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication, July 7, 1930)

I

In many freshly gathered cells of *Valonia ventricosa* the apparent resistance of the protoplasm to direct current varies with the value of the potential applied to measure it.[1] It was further shown[1] that the apparent resistance of a single layer of protoplasm in such cells depends also upon the direction of the current, falling somewhat when the applied potential is increased to carry the positive current outward across it, but rising greatly during the first increase of 100 mv. in the opposite (inward) sense.

These directional effects were studied in cells in which the contact-region at one end had been killed with chloroform. They were therefore limited to the time during which the intact end remained uninfluenced by the diffusion of chloroform, or by the death of the other end. This was at most 15 minutes to half an hour. In order to extend greatly the time of these measurements across a single layer of protoplasm, and to avoid the influence of toxic agents, a series of resistance measurements was made with *V. ventricosa* cells impaled upon fine glass capillaries. Through these the current was led into (or out of) the vacuole, and passed but once across the protoplasm to (or from) the surrounding sea water.

It is recognized that the advantages of this method are balanced by the possible injury inflicted by the insertion of a capillary into the cell. It has already been noted[2] that cells which have reached the

[1] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 793.

[2] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 361.

"constant" state may be rendered again "variable" (*i.e.*, having an apparent resistance depending on the value of the applied potential), simply by impalement. However, the production of this state at will has contributed toward an understanding of its causes, and it is believed that an objective statement of the changes occurring as the cells remain impaled may be valuable. The formation of an "electrical seal" about the capillary has already been referred to in the static measurements of Osterhout, Damon, and Jacques.[3,4] The present measurements of resistance and polarization during the flow of current across the whole protoplasmic film yield further criteria on the condition of impaled cells.

II

The present technique was directly adapted from that of Osterhout, Damon, and Jacques, the impalement being done under a slight pressure and a small hydrostatic head subsequently maintained to prevent the collapse of the cells. Various arrangements utilized are shown in Figs. 1, *a*, *b*, and *c*. Supported by the capillary above, and by a ring of cork or glass below, the cell was held gently and motion upon the capillary was prevented. This immobility contributed largely to the prompt recovery and long life of the cells. The use of rather small cells (from 0.5 to 1 cm in diameter) was also helpful, since the protoplasmic film of larger cells seems to be much more unstable, and breaks up into hundreds of tiny cells (inside the old cell wall) very promptly after mechanical injury such as pricking. Smaller cells (2 to 3 mm. in diameter) could sometimes be impaled without support from below (Fig. 1 *d*).

The capillaries usually projected 2 or 3 mm. into the vacuole; they were about 1 cm. long and had an outside diameter of 0.2 to 0.5 mm. They were drawn on the ends of ¼ inch (or smaller) glass tubing, which, filled with artificial sap of *Valonia ventricosa*,[5] formed the salt-bridge connecting the vacuole to one of the electrodes.

[3] Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927–28, **11**, 193.

[4] Damon, E. B., *J. Gen. Physiol.*, 1929–30, **13**, 207.

[5] This had the composition: 0.6 M. KCl, 95 parts, to 0.6 M. NaCl, 5 parts. For the original analysis of this sap see Cooper, W. C., Jr., and Blinks, L. R., *Science*, 1928, **68**, 164.

In this they resemble most of the so-called "micro-electrodes," which are generally microscopic only as respects the capillary point inserted into the cell, and are more properly "micro-salt-bridges" connecting the cell interior to more or less distant electrodes of quite ordinary size.[6] This is indeed a more satisfactory arrangement than a minute metal surface, in any electrical measurement where polarization is to be avoided; in the present studies particularly, where current flow is purposely caused in order to produce the described effects, such polarization

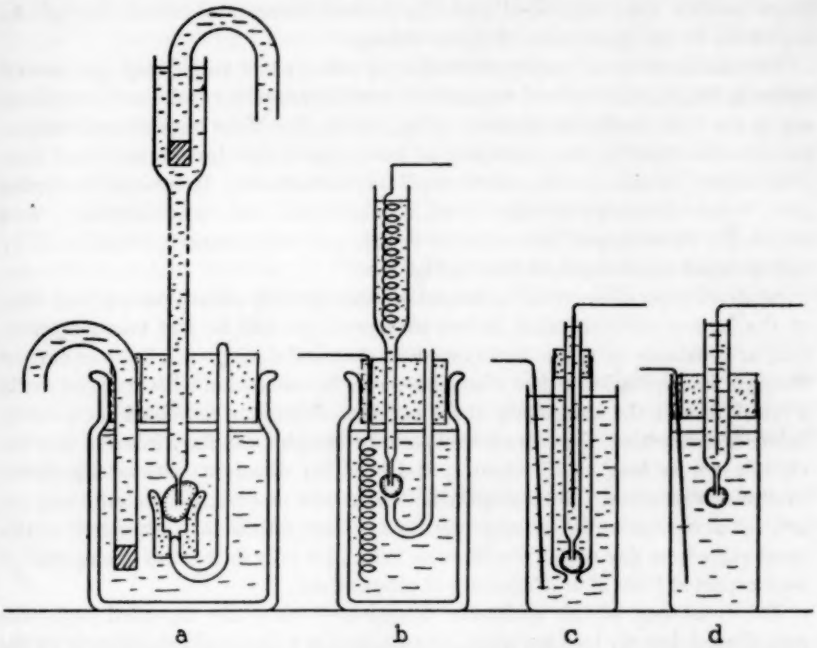

FIG. 1. Apparatus used for supporting the impaled cells of *Valonia*. In each case the capillary is drawn on the end of a vertical tube which fits rather tightly in the cork stopper. It is filled with artificial sap. In *a* the cell is supported from below on a cork with flexible prongs, itself supported on a glass rod inserted firmly into the cork stopper. In *b* the support is the flared end of a glass tube similarly inserted. In *c* the cell is supported inside the narrow orifice of a wider glass tube. In *d* there is no lower support for the small cell. Except in *a* the electrodes are of straight or coiled silver wire, inserted into the sea water or artificial sap as shown. In *a* there are agar-plugged salt bridges connecting to lead electrodes (not shown).

[6] But the silver plated quartz needle recently described by Sen is really a micro-electrode. Sen, B., *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27**, 310.

is inadmissible. It was found that very small electrodes of fine silver wire, chloride-coated and introduced through the capillary into the vacuole of impaled cells, gave very erratic results and seriously distorted the observed effects. There was also the danger of injury to the protoplasm by minute amounts of silver or other salts dissolving from metal electrodes inserted directly into the vacuole for long periods of time, and the impossibility of cleaning and recoating the surface at frequent intervals while in the cell. Instead, more distant electrodes of much larger surface were employed and the desired currents obtained through the capillaries by the application of higher voltages.

The electrodes were usually of straight or coiled silver wire (20-gauge) several inches in length, and inserted respectively into the sea water and into the artificial sap in the tube above the capillary (Figs. 1 *b*, *c*, *d*). They were cleaned and recoated with chloride electrolytically at least once a day (or oftener when their polarization, measured with a blank capillary, warranted). In some of the studies even larger electrodes of sheet lead, amalgamated and chloride-coated, were employed; these dipped into separate vessels and were connected to the cell by agar-plugged salt-bridges, shown in Fig. 1 *a*.

At either type of electrode, polarization was virtually absent during long flows of the largest currents used in the measurements (50 to 100 microamperes). Similar constancy of ohmic resistance was observed during such flows of current through the capillaries, either when these were in connection with artificial sap in a vessel, or with the natural sap of the vacuole. Thus in a cell killed immediately before measurement, the current was passed through a capillary inserted into the vacuole, for as long as 15 minutes, and in either direction, without significant change of resistance. Such constancy is of course essential for determining the amount of change in the living protoplasm. Very satisfactory agreement of this basal value from day to day was likewise found, the only fluctuations being such as were expected from 2° or 3° changes of temperature.

The constancy of this resistance disappeared when the sap-filled capillaries were dipped directly into sea water, or remained in a dead cell long enough for the sea water to diffuse into the vacuole. In these cases the resistance rose when the positive current was passed inward, and reached a value about 20 per cent higher when most of the K^+ ions had been supplanted in the capillary by the less mobile ions of the sea water. Such a diffusion of sea water into the sap is a sign of gross injury, and this effect was never found in healthy cells. Since the rise of resistance in the capillary is entirely ohmic, it is easily distinguished (by the absence of back E.M.F.) from the protoplasmic phenomena, which will later be shown to be largely reactive (i.e., due to the production of a back E.M.F.).

Further check on the basal resistance of the capillary was given by alternating current measurements at a frequency (5000 cycles) where the impedance of the protoplasm was negligible. These gave very constant readings from day to day, independent of the changes of apparent resistance to direct current occurring in the protoplasm.

Depending upon the diameter and length of the capillary, this basal resistance (S) ranged from 10,000 to 100,000 ohms. In order to pass sufficient current through such capillaries to obtain the described effects in the protoplasm it was sometimes necessary to apply as much as 4 or 5 volts to the electrodes. The potential drop across the protoplasm was, of course, but a small fraction of this. The total applied potential (V), and the apparent direct current resistance (R), were determined in the Wheatstone bridge previously described.[2] This bridge also provided for the measurement of potential differences existing in the cell, either by compensating to zero with a series potentiometer, or by deflection of the direct reading detector (vacuum-tube electrometer with string galvanometer). This could be done at full sensitivity, with one arm of the bridge thrown out (open circuit), or, in the condition of balance, *with sensitivity reduced one-half*.

The values of net protoplasmic potential difference were obtained by subtracting or balancing out the P.D. between the electrodes, due to the different chloride content of sea water and artificial sap. This P.D. was usually about 4 mv.

For purposes of comparison with previous papers,[1,2] the results with *Valonia ventricosa* are here described. The cells were used in June, July, and August at Dry Tortugas, Florida, at temperatures between 25° and 30°C., and later in New York at lower temperatures with good agreement of the results.

Further general agreement has been found in the behavior of impaled cells of *V. macrophysa* in the course of continued study at Bermuda, except that these cells show even larger polarization potentials, and recover more readily to a condition resembling the "constant" state of intact cells. These are reserved for discussion in a later paper.

As before, it is a pleasure to acknowledge the generous co-operation of the Carnegie Institution of Washington in offering the facilities of its laboratory at Tortugas.

III

This section describes the characteristic values of potential difference naturally existing across the protoplasm of *Valonia ventricosa*. As indicated in a preliminary report,[7] these differ somewhat from those of *V. macrophysa* as described by Osterhout, Damon, and Jacques.[3,4] Theoretically the values of bioelectric potential, developed by the protoplasm in contact with different solutions, should have a close relation to the polarization potentials elicited by the flow of current to or from such solutions. This expectation has been justified in the correlation found between several types of polarization responses and the bioelectric potential which accompanies them.

[7] Blinks, L. R., *Carnegie Inst. Washington Year Book*, 1928–29, **28**, 277.

We may indicate five typical stages of the bioelectric potential difference:

(1)

Initial mechanical shock—P.D. 5 mv. or less (inside positive).

This stage follows immediately after impalement and lasts from a few minutes to several hours, depending apparently on the degree of injury sustained. The earliest measurable P.D. (some 10 seconds after impalement) is about 5 mv.; this usually drops within a few minutes to nearly zero. There is then a slow recovery of 1 or 2 mv. per hour, this process passing into stage (2) without apparent break. But in occasional cases there may be a complete reversal of P.D. to about 30 mv. (*outside positive*). This usually occurs promptly after impalement, lasts but a few minutes and is as promptly recovered from. It seems to occur most frequently in cells which have already reached the "constant" state before impalement, and it is really a momentary example of stage (4), probably elicited by the breakdown of the outer layer (X) of the protoplasm.[4] As X recovers or Y in turn breaks down, the P.D. returns to zero and the remaining slow recovery process ensues.

It may be noted that the potential differences given for stage (1) are in good agreement with those observed by Taylor and Whitaker[8] soon after the insertion of a micro-electrode into the vacuole of *Valonia ventricosa*. They state that there was "a relatively rapid lowering of the potential difference, which was then followed by a gradual rise to a maximum . . . . Although the amount of voltage varied for different cells, we have estimated a mean value of the potential difference between the cell sap of *Valonia* and sea water to be about 0.002 volt. The charge of the cell sap of *Valonia* was plus with reference to sea water." Had they followed the process longer there can be no doubt the P.D. would have been found to rise to values typical of stage (2). The necessity of such long waits for impaled cells to become stabilized was insufficiently realized for a long time, and probably also accounts for the low values (1 or 2 mv.) reported by Osterhout,[9] who first measured the P.D. in this manner in *Valonia*, and by Jost[10] who repeated them. Umrath[11] has recently stated that his earlier reports for *Nitella* were likewise too

[8] Taylor, C. V., and Whitaker, D. M., *Carnegie Inst. Washington Year Book*, 1925–26, **25**, 248.

[9] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–26, **7**, 561.

[10] Jost, L., *Sitzber. Heid. Ak. Wiss.*, 1927, Abh. 13, 1.

[11] Umrath, K., *Protoplasma*, 1930, **9**, 576.

low for the same reason. This need of recovery was first pointed out by Osterhout, Damon, and Jacques.[3] In early experiments of the author to measure the electrical resistance of impaled cells, great ambiguity was met through similar neglect of the time factor, and the method was temporarily abandoned.

(2)

The stage of recovery and normal potential: P.D. from 8 to 25 mv. with an average of 15 mv. (inside positive).

This is reached within several hours to 2 days after impalement, and persists through the life of the cell, with a gradual decrease of value.

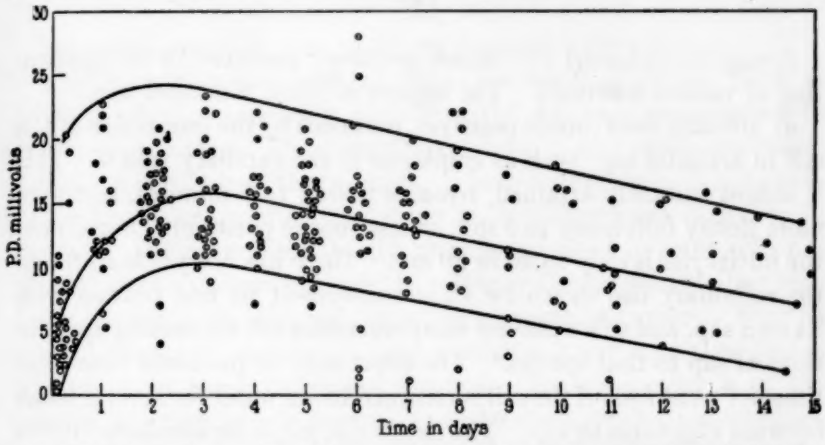

FIG. 2. Composite graph of the P.D. measured across the protoplasm during the life of 34 impaled cells of *Valonia ventricosa* in sea water. Each point is a single determination. The outer lines roughly enclose nearly all the scattered values, and the median line is drawn freehand to represent an average value. The P.D. is that of the vacuole, which is positive in the electrometer circuit to the sea water.

Fig. 2 gives a composite picture of the course of these values for a representative group of cells. The variations from day to day might be several millivolts, but in general a given cell had a more or less constant P.D., and those which were apparently most healthy (*i.e.*, dark green, turgid, and shiny) gave the highest values.

It should be noted that this P.D. is in the same direction as that of *V. macrophysa* similarly impaled and immersed in sea water, but has a normal value from 2 to 3 times as great. This is a specific difference, which if connected only with the higher potassium content of the sap

of *V. ventricosa*, must argue *against* a high mobility of K^+ ion in the protoplasm, at least at the inner surface, since the P.D. is increased in the wrong direction, the positive current tending to flow from sea water *inward* to the sap. The difference can probably be explained on the basis of 3 layers in the protoplasm.[4]

While stages (1) and (2) are the only ones necessarily passed through by the living impaled cells, there are two other stages which may temporarily be produced by special causes, and have important relations to the polarization phenomena. These are:

(3)

A stage of increased P.D. (inside positive), produced by the application of various solutions. The highest of these measured was:

a) 100 millivolts (inside positive), produced by the immersion of the cell in artificial sap (such as employed in the capillary tube[5]). This is almost instantly attained, remains from 1 to 5 minutes, and then more slowly falls away to 5 mv. or less (inside positive). Sometimes the initial rise is only to 40 or 50 mv. There has not yet been found the secondary rise shown by *V. macrophysa* at its first contact with its own sap, and this behavior more resembles the succeeding applications of sap to that species.[4] The effect may be produced time after time in *V. ventricosa* if the cell is returned to sea water for several hours between exposures to sap. The rise of P.D. might be attributed to the high mobility of the K^+ ion in the *outer* surface of the protoplasm, and its succeeding fall to the total imbibition of the latter with KCl destroying the gradient of K^+ concentration across the outer surface. Protoplasmic asymmetry might thus be said to exist after the exposure of cells to sea water, but to disappear upon their imbibition with sap. According to Damon's explanation[4] for the shape of curves in successive applications of sap to *V. macrophysa*, the middle layer (*W*) of the protoplasm in *V. ventricosa* might further be considered always fairly well imbibed with KCl. 0.6 M KCl acts much like sap in these experiments.

b) In addition to the large temporary increase of P.D. produced by sap, other, smaller, increases were produced by immersion of the cells in diluted sea water (1/10 sea water to 9/10 isotonic glycerine), and in 0.6 M $MgSO_4$. In each case the P.D. rose to 35 or 40 mv. (inside

positive) and remained up for half an hour or longer. This increase is very much like the effect of dilute sea water on *V. macrophysa*.[12] The direction of this increase argues for a higher mobility of some anion, presumably Cl^-, in the inner surface of the protoplasm, since the gradient is greatly increased by these treatments. Immersion of the cells in 0.6 M NaCl promptly brings the P.D. nearly to zero, where it remains, and this could be ascribed to the abolition of the Cl^- gradient.

We now come to a fourth typical and very interesting stage:

(4)

Reversed potential: P.D. to 50 mv. (*outside* positive).

This frequently occurs after a long exposure to dilute sea water or $MgSO_4$ solution, especially just after the cells are re-immersed in ordinary sea water. The change to this level is very rapid, sometimes being completed in a few seconds. The recovery from it is usually somewhat slower, and its total duration is seldom more than 5 or 10 minutes. Its occurrence shortly after impalement in some cells has already been noted under stage (1). It may also be elicited after very large current flows, *either* of inward or of outward current. For the latter it is not in the direction of a counter E.M.F. and therefore is distinct from the polarization response. Its independence of the current direction, and its production under a variety of circumstances, suggests rather that it is in the nature of an "alteration" such as the temporary destruction of the outer protoplasmic layer $(X)^4$ (or the reduction of its E.M.F.), leaving the outwardly directed E.M.F. of Y. This alteration is not permanently injurious to the cell, and is indeed characteristically found in those cells most nearly "normal" or "constant" in their apparent resistance. Thus it is most frequently seen just after "constant" cells are impaled, and then again toward the end of stage (2) when the cells have recovered from the injury of impalement. The ease of its production may then actually become troublesome in the measurement of bioelectric potentials.

[12] Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 445.

(5)

The abolition of potential: (P.D., 1 mv. or less).

This stage is reached on the death of the cell even before the protoplasm is obviously disintegrated. It may be produced by long applications of single salt solutions, or by heat, or by chloroform and other toxic agents. It is of no particular biological interest therefore, but serves as a check on the electrodes and on the experimental technique.

IV

We may now turn to the phenomena caused by the flow of current at these different stages.

Stage (5) has already been referred to in the discussion of methods, and serves as a convenient check upon the constancy of the electrodes and of the capillary resistance. In impaled cells just killed by chloroform, as much as 5 volts may be applied to the electrodes, and an approximately constant resistance measured during as long as a half hour of current flow in either direction. Neither electrodes, capillary, dead protoplasm nor cell wall introduce any significant change of resistance during this time. This shows that the heating effect of the current is negligible, and that migration of sea water into the vacuole is small.

In stage (1) are found rather similar conditions, except that the resistances tend to be slightly higher than those of the blank capillary before its insertion, and there is a feeble reaction, shown in the detector as a small back E.M.F. at the break of the current (of either direction). Occasionally there is a slightly higher apparent resistance to a small outward current than to an inward current, especially in cells where the reversion to stage (4) occurs. In general the effective resistance of the freshly impaled cells is so low as to be negligible at all current densities. This unexpected result in the early experiments of the author led to the temporary abandonment of the capillary method, since it could not be reconciled with the high effective resistance of the same cell measured just before its impalement. The low resistance is almost certainly due to leaks around the insertion of the capillary, but also to more profound changes in the entire protoplasmic surface. The nature of these changes begins to appear in the recovery process.

Stage (2). If now, after the cell had been impaled several hours, or as much as a day, a small potential (0.25 to 0.5 volt) was applied to the electrodes, the behavior was even more like that of stage (5) or a dead cell. The small reaction (back E.M.F.) shown in stage (1) had usually disappeared, and the resistance was essentially equal to that of the blank capillary before its insertion (or to the alternating current resistance measured at the moment). When the potential was increased in a direction to carry the positive current *outward* (from sap to sea water) across the protoplasm, this low resistance was maintained, even up to currents of 100 microamperes (caused by the application of 5 volts to a capillary of 50,000 ohms resistance).

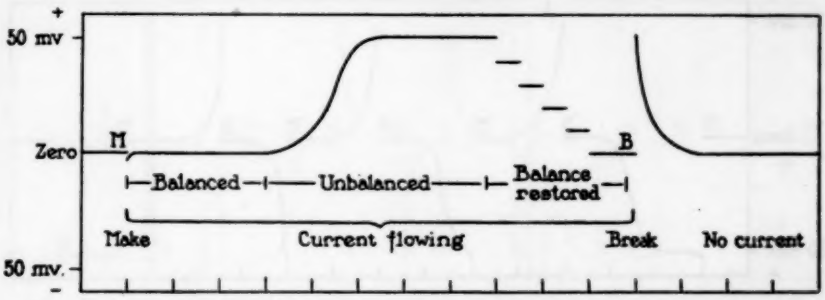

FIG. 3. Tracing from string galvanometer record showing deflections of the bridge detector during the flow of current inward across the protoplasm of an impaled cell of *Valonia ventricosa*. The upward movement is due to a rise of resistance, or to the appearance of an E.M.F. opposed to the applied potential. After the rise has occurred R is increased in 5 steps of 1000 ohms each to bring the detector to zero. At break there is a momentary upward movement due to the back E.M.F. R = 45,000 ohms, later increased to 50,000 ohms. Applied potential, 1 volt. Detector sensitivity in open circuit as shown. Time marks, 1 second. M, make; B, break. (P.D. of cell was compensated to zero before the record.)

Only when the current was reversed and passed *inward* across the protoplasm from sea water to sap was there a change of the apparent resistance. At slightly higher voltages, between 0.5 and 2 or more volts in these early stages, this change occurred rather suddenly (Fig. 3). At the critical voltage there was at first no response to the flow of current, but in from 1 to 20 seconds there began a rise of apparent resistance, at first slow, then very fast, then slower again, sometimes carrying the image of the galvanometer fiber out of the

field. When this was brought back to the center by balancing the decade resistance box of the adjacent bridge arm, a steady state was now maintained as long as the potential was applied to the bridge. The amount of the rise varied from 1 or 2 per cent up to 10 per cent or more of the capillary resistance, and sometimes as much as 10,000 ohms additional resistance was required in the decade box to balance this apparent rise. What now occurs at the break of current, when the potential is removed from the bridge input terminals? In an ordinary resistance balance, through a wire resistance or through a blank capillary, the galvanometer image being brought to zero remains there

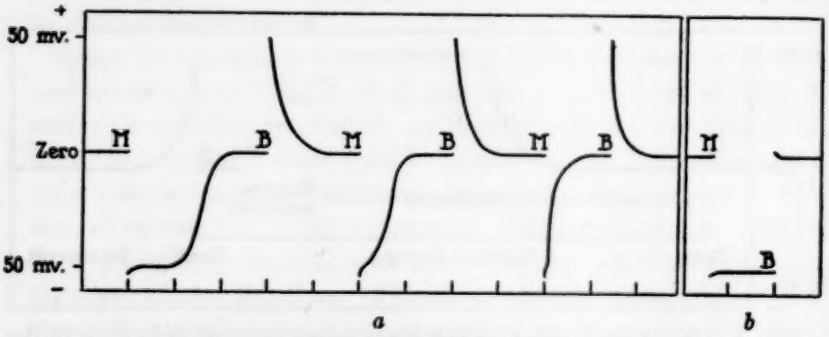

FIG. 4. Tracing from string galvanometer record showing deflections of the bridge detector during applications of 1 volt to the cell of Fig. 3. R = 50,000 ohms throughout. *a*, inward current, 3 applications. *b*, outward current. Detector sensitivity in open circuit as shown. Time marks, 1 second. *M*, make; *B*, break. (P.D. of cell was compensated to zero before the record.)

when the potential is removed from the bridge. But in this case the image deflected greatly at the instant the outside potential was removed, and then returned slowly to zero again. Herein the cell resembled a platinum electrode, or a large condenser, and the deflection at break was due to the accumulated charge, or polarization potential. This is shown in Fig. 3, together with the preceding rise of apparent resistance and the balance to zero.

If, now, the decade box increment being retained and the bridge balance kept at the steady state value reached in Fig. 3, we apply the same critical voltage again, the record of Fig. 4 ensues. A rather long wait has followed the "break" of Fig. 3, so that the string image

is found now to be deflected approximately as far downward as it previously had risen upward. There is a delay of about a second at this level, and then the rise begins again, at first slowly, then more rapidly, finally flattening out at the center (zero) line representing the bridge balance. At removal of the potential from the bridge ("B") there is again an upward deflection, due to the back E.M.F. When this has returned fully to zero for about a second, the potential is again applied to the bridge and again there is a deflection downward, which is much more rapidly recovered from, but still shows the double inflection of the first curve. Again there is an upward deflection at

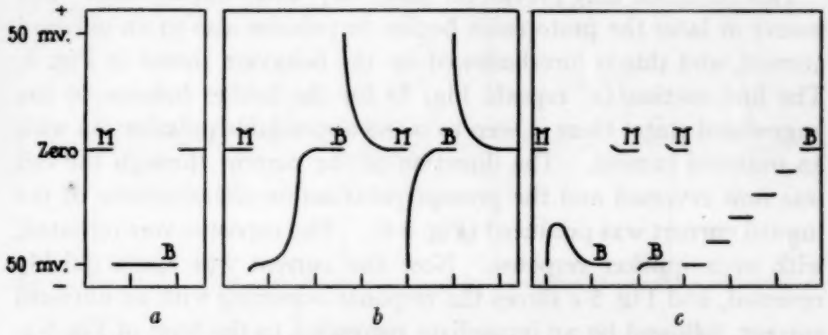

FIG. 5. Tracing from string galvanometer record showing deflections of bridge detector during applications of 1 volt to cell of Figs. 3 and 4, at a partially recovered state. *a*, Outward current; *b*, inward current; *c*, outward current. In the latter the first exposure causes a partial polarization, followed by a regression. R = 50,000 ohms until the 3rd exposure in *c*, when it is reduced in 1000 ohm steps to 45,000 ohms. Detector sensitivity in open circuit as shown. Time marks, 1 second. *M*, make; *B*, break.

"break," returning smoothly to zero. A third application after another second's delay gives a smooth curve which has almost no hint of a double inflection and is nearly an image of the return curve at break. It now closely resembles the charge and discharge curves of a condenser connected in the bridge, or the polarization curves of a blank platinum electrode. It is evident that an effect of previous flow of current persists from one exposure to the next, and that there has been a progressive change in the response of the protoplasm suggesting that of a change from a reversible electrode to a polarizing

electrode. This however, holds only for *inward* currents, and if the current be reversed through the cell we obtain the record of Fig. 4*b*. The galvanometer image deflects immediately downward, and stays there as long as the current flows. The downward deflection is due to the maintenance of the bridge balance at the augmented value of R caused by the inward flow of current. If the balance be returned to that of the basal resistance S, the string image returns to zero, and there are no appreciable polarization phenomena either at make or break (*i.e.*, the record is essentially a straight line). The protoplasm is apparently a perfectly reversible electrode to an outward current.

This condition may prevail for many days after impalement. But sooner or later the protoplasm begins to polarize also to an outward current, and this is foreshadowed by the behavior shown in Fig. 5. The first section (*a*) repeats Fig. 4*b* for the bridge balance to the augmented state; there is seen to be no appreciable polarization with an outward current. The direction of the current through the cell was now reversed and the prompt polarization characteristic of the inward current was produced (Fig. 5 *b*). The exposure was repeated, with even quicker response. Now the current was again quickly reversed, and Fig. 5 *c* shows the response occurring with an outward current, followed by an immediate regression to the level of Fig. 5 *a*. On break there is little or no back E.M.F., the image returning directly to zero without an excursion above the line. On the second application there is not even a temporary polarization, and the deflection is instant and complete. Finally the record shows the restoration of the bridge balance to the basal value (S) in steps of 1000 ohms.

It is evident from this record that the protoplasm is still not ordinarily polarizable to an outward current, but may be made so temporarily. Either by the movement of ions out of the protoplasm into the vacuole, or by the alteration of the protoplasmic properties, the previous inward current has left an effect which persists a short time. The further recovery process in the course of stage (2) has the same effect as this flow of current and Fig. 6 shows the response given at a later time by a cell that is beginning to polarize normally with currents in either direction. Fig. 6 *a* shows the prompt and symmetrical charge and discharge curves with the inward current. Fig. 6 *b* shows nearly an equal response with an outward current, but there is a succeeding

regression which begins at a lower potential than with the inward current. This in general represents the behavior of the cells for the remainder of their life upon the capillary. They give good polarization with as low a potential as can be applied, and, for values up to about 0.5 volt (depending upon the resistance of the capillary) have a nearly constant apparent resistance with the current either direction. Above this value the apparent resistance may fall off, more rapidly with outward currents than with inward ones. The breakdown process is much the same in either case, being a regression of polarization following a preliminary rise, and simply occurs at lower

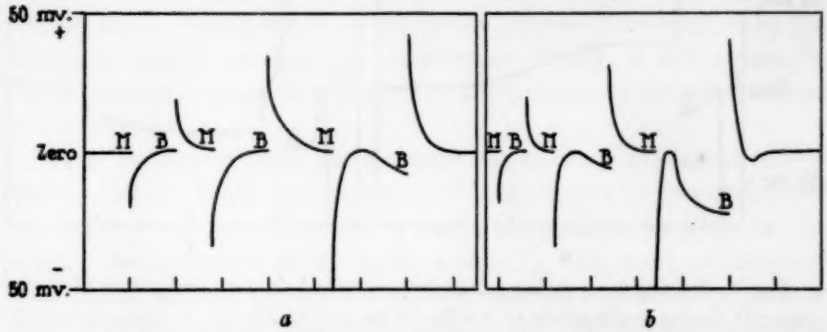

FIG. 6. Tracing from string galvanometer record showing deflections of bridge detector during applications of increasing voltages to an impaled cell of *V. ventricosa* which has reached the "constant" state. Applied potentials: 0.5, 1.0, and 1.5 volts, in the order given. *a*, Inward current; *b*, outward current. Detector sensitivity as shown (open circuit). Time marks, 1 second. *M*, make; *B*, break.

current densities in the outward direction than in the inward. In this the cells in the recovered condition of stage (2) resemble "constant" intact cells which have been chloroformed at one end, and are considered to have reached as nearly normal a state as the impaled cells ever do, having an apparent resistance that is independent of the applied potential up to the breakdown value.

In stage (3), the condition of increased P.D. across the protoplasm, the relations of stage (2) still hold quite well. Most remarkable of all, the polarization response obtained when the cell is immersed in artificial sap is about as great as when in sea water, and is characteristically even more rapid in its development, having a very abrupt

rise. After the exposure to sap has been continued for some time the polarization becomes less, and eventually may even be larger with an outward current than with an inward one. But for as long as half an hour after some cells have been immersed in artificial sap, their polarization response remained large and their apparent resistance as high as in sea water. It is difficult again to reconcile this result with a high mobility of K^+ ion across the entire protoplasm, and it may become necessary to ascribe the effects to some unspecified ion, possibly

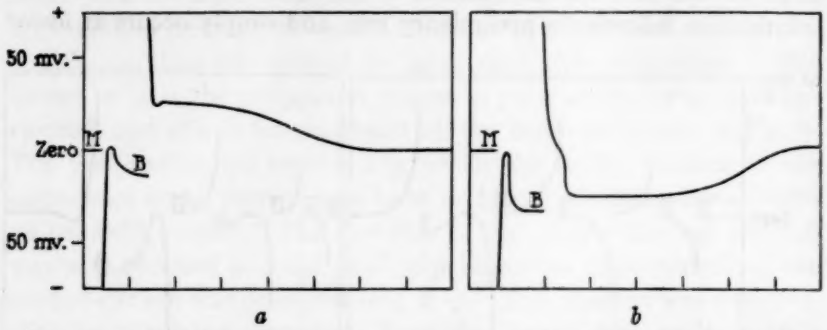

FIG. 7. Tracing from string galvanometer record showing deflections of bridge detector during applications of 3 volts to the cell of Fig. 6. *a*, Inward current; *b*, outward current (in *b* the cell is reversed in bridge). In each case the appearance of the polarization E.M.F. at break is succeeded by stage (4) with a reversed P.D. of about 50 mv. (outside positive). R = 38,000 ohms; open circuit sensitivity of detector as shown. Time marks, 1 second. *M*, make; *B*, break.

existing only within the protoplasm, and not dependent upon the environment immediately supplied in the experiment.

Finally we come to the anomalies of stage (4). These consist both in its production, and in the polarization phenomena which occur when it is established. Its production mechanically (by impalement) and chemically (by dilute sea water, etc.,) has been described above. Fig. 7 shows its production after the passage of large currents; in *a* after an inward, in *b* after an outward current. After the inward current, it has the same direction as the polarization potential and may normally add to the value of this. Certainly some frequent distortions of the return curve may be ascribed to a momentary production of this stage. But in the present case it forms a very

distinct level equal to about 50 mv., which persists for several seconds before returning to zero. In Fig. 7 *b*, after a large outward current has passed, there is first a large upward excursion due to the normal back E.M.F., but as this returns to zero there is a further movement, and the image goes below the zero line, again to an amount representing about 50 mv. This is now contrary to the polarization E.M.F., and is therefore in the same direction as the applied potential, which had driven the current *outward* across the protoplasm. The P.D. of stage (4) is thus in reverse of the normal potential and is *outside positive* to the electrometer. (In Fig. 7 *b* this P.D. appears in an opposite direction from Fig. 7 *a* because the cell has been reversed in the bridge.) Whether it is caused by the outward flow itself, or by the return current from the large polarization charge, is still uncertain. In either case it could be explained by the temporary alteration of the outer layer (*X*).[4]

While in these records the reversed P.D. of stage (4) lasted only a few seconds, in other cases it remained long enough for measurements of effective resistance to be made across the protoplasm while in this stage. As indicated before under stage (1), the usual polarization phenomena are now reversed, and the resistance appears to be greater to an outward current than to an inward one. This continues as long as the reversed P.D. remains, and then, as it begins to return to normal, polarization becomes more nearly equal in both directions and finally reaches that of the late stages of (2).

Study of the records has shown that the P.D. of stage (4) is frequently driven approximately to zero by an outward current. Since this upward movement is characteristic of a back E.M.F., it would therefore appear to be a polarization, and would be measured as an increased resistance. On the other hand, the P.D. of stage (4) is but little increased by an *inward* current, and there is almost no back E.M.F. developed in excess of the existing P.D. (which, of course, is taken as the null point, either balanced out or used as a pseudo zero). Thus an apparent polarization of as much as 40 or 50 mv. to an outward current may really be due to the destruction of so much P.D. This illustrates a pitfall inherent in these measurements, which may have to be taken account of in other biological "polarizations." That it is not entirely responsible for the total polarization to an outward current

has, however, been satisfactorily shown in *Valonia macrophysa*, and will be considered more fully in a paper dealing with that species. Since an increased polarization to an outward current sometimes appears during the exposure of cells to artificial sap, when there is no reversal of P.D. across the protoplasm, further interest is added to its study.

One question may remain concerning the recovery of impaled cells to a "constant" state. May not the polarization apparently occurring with currents directed outward really be due to their passage inward across a film of protoplasm formed over the orifice of the inserted capillary? Aside from the difference of constant intact cells which have been chloroformed at one end, and which show much the behavior of recovered impaled cells, there is the great difference of current densities between the point of the capillary where the current enters the vacuole and over the general surface by which it leaves. The area of protoplasm formed over the end of the capillary could not be more than 1 mm.^{2} while that of the cell surface is usually from 2 to 3 cm.^{2}—a more than thousandfold difference. It is practically assured therefore that the relatively immense current density—a thousandfold greater—across the smaller protoplasmic film, if any, would break this down and render its resistance and reactance negligible. A ten to hundredfold increase is sufficient to do this on the remaining surface, as will be shown in a later paper dealing with injury and recovery. There is also the fact that such a protoplasmic plug would be bathed on its outer surface with artificial sap from the capillary, which is quite sufficient to destroy the protoplasmic properties of normal cells after exposures of a day or two.[9]

Still better evidence that such a film is not concerned is fortunately available, due to the straight open tubes employed in connection with the capillaries. With cells which had recovered to the "constant" state a fine platinum wire (attached on the end of a glass rod) was carefully inserted from above into the capillary, and a short distance into the vacuole. This was moved up and down gently and must have broken any plug which had formed. When the measurements were now repeated immediately after this probing the polarization effect was the same as before, and perfectly representative of the "constant" state as defined.

V

It has already become sufficiently evident that the apparent rise of resistance in the impaled cells is not real or ohmic, but is due to a polarization potential. This, decreasing the current through the system by opposing the applied potential, is therefore measured as increased resistance. The reality of this back E.M.F. is evident from the string galvanometer records, and its responsibility for practically

all of the apparent resistance rise is shown by the essential constancy of the alternating current measurements throughout the life of the impaled cells, independent of their effective resistance to direct current.

This being so, the value of the polarization potential (in millivolts) becomes of more interest than the apparent resistance rise as measured in ohms. Several ways of determining this—by direct reading in a calibrated electrometer, by compensation with an opposed series potentiometer, or by measurement of the galvanometer deflection at the instant of break, as recorded photographically—have been employed, and will be reported upon in the paper dealing with *V. macrophysa*. These methods all agree well with the calculation of the back E.M.F., (P), from the known values of the applied potential (V), basal resistance (S), and apparent resistance (R), in which form the data for *V. ventricosa* were mostly recorded.

Thus in Fig. 3 where

V = 1.0 volt
S = 45,000 ohms and
R = 45,000 ohms (at the initial balance),

there is at first no back E.M.F. and hence no deflection of the detector. As the back E.M.F. builds up, the detector deflects upward, and reaches an apparent value of approximately 50 mv. Since the sensitivity of the detector to a P.D. in the cell is reduced one-half with the bridge closed,[2] this P.D. is therefore about 100 mv. The polarization potential appearing at "break" is also seen to go momentarily to this value.

When now the bridge was balanced to the augmented steady state, in the five steps of 1000 ohms each, shown in Fig. 3.,

V = 1.0 volt
S = 45,000 ohms, but
R = 50,000 ohms

By the equation

$$P = \frac{R-S}{R} \cdot V$$

the polarization potential $P = \frac{5000}{50{,}000} \cdot 1.0 = 0.1$ volt or 100 mv., which agrees with the directly observed potential.

TABLE I

Value of Polarization Potential in the Protoplasm of Impaled Cells of Valonia ventricosa. (*Calculated from Apparent Resistance to Direct Current*)

Stage	Description	P.D. of cell before current flow		*V* Applied potential causing current:		*S* Basal resistance of capillary, etc.	*R* Apparent direct current resistance of capillary plus cell	*P* Back E.M.F. of protoplasm
		In +	Out +	Inward	Outward			
		mv.	*mv.*	*volts*	*volts*	*ohms*	*ohms*	*mv.*
1	Immediately after impalement	3.0		0.5		46,600	46,600	0
				1.0			47,000	8.5
				2.0			46,900	8.5
					0.5		46,700	1.0
					1.0		46,900	6.4
					2.0		47,000	17.0
2	Shortly after impalement	20.0		0.5–1.5		26,200	26,200	0
				2.0			27,500	95
				2.5			27,200	92
				3.7			26,900	94
					0.5–3.7		26,200	0
	Partially recovered	15.0		0.5		22,500	25,000	50
				1.0			25,300	110
				1.5			25,100	155
					0.5		23,000	11*
					1.0–1.5		22,500	0
	"Constant" state	12.0		0.25		35,500	38,000	16.5
				0.5			38,000	33.0
				1.0			38,000	66.0
				1.5			37,500	80.0
					0.25		38,000	16.5
					0.5		38,000	33.0
					1.0		37,500	53.0
					1.5		37,000	61.0
3	In artificial sap 5 minutes	5.0		1.5	1.5	26,200	27,200	55.0
							27,300	60.0
	In artificial sap 20 minutes	1.0		1.5–3.7	1.5–3.7		26,200	0
4	P.D. reversed by dilute sea water			0.25		56,000	56,200	1.0
				0.5			56,100	1.0
			45.0	1.0			56,000	0
					0.25		65,000	34.5
					0.5		61,000	41.0
					1.0		59,000	51.0
5	Cell killed by chloroform		1.0	0.5–3.0	0.5–3.0	45,400	45,400	0

* Polarization occurs at first application only, and is followed by a regression to zero.

Table I gives further values of polarization potentials calculated by this means. It is seen that in the early stages of impalement these values are very small for small applied potentials, but increase suddenly and may reach values of 150 mv. or higher with inward currents. Later, smaller polarization potentials appear with currents of either direction, and for part of the range, are closely proportional to the values of the applied potential. This has the effect of producing a uniform apparent resistance and the cells may be called "constant."

We may now with profit go back to the results with intact cells of *Valonia*,[1,2] and consider their apparent direct current resistance in the light of polarization potentials caused by the flow of current. Always taking account of the cell wall forming a constant shunt around the protoplasm, the apparent resistance of the latter was found to vary from less than 1000 ohms, in the freshly gathered "variable" cells,[1] to over 50,000 ohms in cells which had attained constancy of resistance.[2] The dead resistance on the other hand was usually 50 ohms or less. Inserting this value (50 ohms) as S, the real ohmic resistance in the preceding equation, we find in every case a very large factor to be multiplied into the applied potential (V) to give the back E.M.F. (P). Thus when $R = 1000$ ohms and the applied potential is 20 mv., the back E.M.F., $P = \frac{950}{1000} \cdot 20$, or 19 mv. When, under the application of 100 mv., the resistance of the protoplasm in the variable cells has risen to 10,000 ohms,[1] an even larger factor results, and the back E.M.F., $P = \frac{9,950}{10,000} \cdot 100 = 99.5$ mv. In "constant" cells having an effective protoplasmic resistance of 50,000 ohms under the application of all potential differences from 10 to 100 mv., the back E.M.F.'s therefore so closely approach the applied potentials as to be essentially equal. The fact that these resistances begin to fall off at values between 100 and 150 mv. shows that in the intact cells the maximum polarization potentials are about half these values (since two layers of protoplasm are measured in series). This agrees well with the maximum values of 50 to 75 mv. developed by either inward or outward current in impaled cells which have reached a "constant" state. On the similarity of these potentials should the comparison between intact and impaled cells be based, rather than upon the apparent resistance levels. For it is obvious that while a back E.M.F. of 49.9

mv. can effectively reduce to nearly zero a current through the protoplasm when 50 mv. is applied to an intact cell, such a back E.M.F. developed by the same initial current density in an impaled cell can only partly counteract a potential of one-half volt applied through the capillary. Thus a much larger current will continue to flow in the latter case than in the former, and to these differences of continued current density may be attributed some of the differences found between intact cells and impaled cells. The study of *V. macrophysa* has been particularly directed toward these differences, and the polarization potential will there be further considered in relation to the current density across the protoplasm.

The *quantity* involved in these effects, as distinct from the *intensity* (E.M.F.) of polarization emphasized in this paper, is the *capacitance* of the protoplasm in its function as a condenser acquiring a charge during a flow of current. It early became evident that the capacitance of *Valonia* cells is very large, *i.e.*, the *quantity* of electricity taken to charge them to a given potential is large and hence a long flow of current is required. Otherwise the records of charge and discharge could not be taken with a (relatively) slow-period instrument such as a string galvanometer. The fact that this instrument gives good records of these curves at quite ordinary camera speeds (2 cm. per second) has greatly simplified their study. The time relations will be considered in a forthcoming paper along with the closely related alternating current measurements. But an indication of the quantities involved is given in such curves as are shown in Figs. 3, 4, 5, and 6. The time course of these is often a second or more (as compared to one or two *thousandths* of a second in human skin[13]) and the time constant τ (time for the potential of any part of the curve to change by $\frac{1}{e}$ of its value) may be 0.2 or 0.3 second. Since (when τ is in seconds, r in ohms, and c in farads) $\tau = r\,c$; and r, the total bridge resistance through which the potential is discharged, is usually not over 100,000 ohms, c, the effective capacitance of the protoplasm, is of the order of 2 or 3 microfarads. Thus for a cell of about 1 cm. diameter and some 3 cm.^{2} surface, this is as high as 1 microfarad per cm.^{2}

It is interesting to note that capacitances of this general magnitude

[13] Hozawa, S., *Arch. ges. Physiol.*, 1928, **219**, 111.

were also found at the lower frequencies of alternating current, in experiments with *Valonia* made several years ago,[14] and in later measurements to be published shortly. The figure is also in good agreement with that for red blood corpuscles (0.81 μ F/cm.^{2}) published by Fricke;[15] and, less closely, with that of McClendon[16] (9 μF/cm.^{2}) for erythrocytes; and of Cole[17] for *Arbacia* eggs (1.02 μF/cm.^{2} at 350,000 cycles, or 18 μF/cm.^{2}, extrapolated to 1000 cycles).

SUMMARY

Electrical resistance and polarization were measured during the passage of direct current across a single layer of protoplasm in the cells of *Valonia ventricosa* impaled upon capillaries. These were correlated with five stages of the P.D. existing naturally across the protoplasm, as follows:

1. A stage of shock after impalement, when the P.D. drops from 5 mv. to zero and then slowly recovers. There is very little effective resistance in the protoplasm, and polarization is slight.

2. The stage of recovery and normal P.D., with values from 8 to 25 mv. (inside positive). The average is 15 mv. At first there is little or no polarization when small potentials are applied in either direction across the protoplasm, nor when very large currents pass *outward* (from sap to sea water). But when the positive current passes inward there is a sudden response at a critical applied potential ranging from 0.5 to 2.0 volts. The resistance then apparently rises as much as 10,000 ohms in some cases, and the rise occurs more quickly in succeeding applications after the first. When the potential is removed there is a back E.M.F. displayed. Later there is also an effect of such inward currents which persists into the first succeeding outward flow, causing a brief polarization at the first application of the reverse potential. Still later this polarization occurs at every exposure, and at increasingly lower values of applied potentials. Finally there is a "constant" state reached in which the polarization occurs

[14] Blinks, L. R., Thesis deposited in the library of Harvard University, 1926. Abstract in Summaries of Theses (1926), Cambridge, 1930, 10.

[15] Fricke, H., and Morse, S., *J. Gen. Physiol.*, 1925–26, **9**, 137.

[16] McClendon, J. F., *J. Biol. Chem.*, 1926, **69**, 733.

[17] Cole, K. S., *J. Gen. Physiol.*, 1928–29, **12**, 37.

with currents of either direction, and the apparent resistance is nearly uniform over a considerable range of applied potential.

3. A state of increased P.D.; to 100 mv. (inside positive) in artificial sap; and to 35 or 40 mv. in dilute sea water or 0.6 M $MgSO_4$. The polarization response and apparent resistance are at first about as in sea water, but later decrease.

4. A reversed P.D., to 50 mv. (outside positive) produced by a variety of causes, especially by dilute sea water, and also by large flows of current in either direction. This stage is temporary and the cells promptly recover from it. While it persists the polarization appears to be much greater to *outward* currents than to inward. This can largely be ascribed to the reduction of the reversed P.D.

5. Disappearance of P.D. caused by death, and various toxic agents. The resistance and polarization of the protoplasm are negligible.

The back E.M.F. of polarization is shown to account largely for the apparent resistance of the protoplasm. Its calculation from the observed resistance rises gives values up to 150 mv. in the early stages of recovery, and later values of 50 to 75 mv. in the "constant" state. These are compared with the back E.M.F. similarly calculated from the apparent resistance of intact cells.

The electrical capacitance of the protoplasm is shown by the time curves to be of the order of 1 microfarad per $cm.^2$ of surface.

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